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# CONGENITAL MALFORMATIONS INDUCED IN RATS BY MATERNAL VITAMIN A DEFICIENCY

## II. EFFECT OF VARYING THE PREPARATORY DIET UPON THE YIELD OF ABNORMAL YOUNG <sup>1</sup>

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FOUR FIGURES

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Congenital anomalies in the offspring of vitamin A deficient animals have been described repeatedly. The literature on this subject has been reviewed by Warkany and Schraffenberger ('46). The studies of Hale ('33, '35, '37) are of great importance, since genetic factors could be ruled out as the cause of the malformations obtained. Hale fed gilts of known stocks a diet deficient in vitamin A and possibly in other nutritional factors. The offspring showed anophthalmos or microphthalmos, accessory ears, harelip and cleft palate, subcutaneous cysts and misplaced kidneys. For experimentation on a large scale the production of congenital malformations by maternal vitamin A deficiency in a small laboratory animal appeared desirable. Anderson ('41) reported briefly the occurrence of diaphragmatic hernia in about 25% of the young of rats bred on a diet containing the minimum amount of vitamin A required for pregnancy and parturition. A small number of the controls which were fed halibut liver oil, showed the same lesion.

<sup>1</sup> This work was aided in part by a grant from the Nutrition Foundation, Inc., New York.

Warkany and Schraffenberger ('44, '46) reported congenital anomalies of the eyes in the offspring of rats that had been depleted of vitamin A. Female rats were raised on a preparatory diet (diet U) which contained small measured amounts of carotene. The carotene supplement was necessary to make possible growth and maturation of the females; however, it was insufficient to provide vitamin A for storage. When the female rats reached a weight of 150 to 160 gm and had regular oestrous cycles they were given a purified diet completely free of vitamin A and carotene (diet W). While on this diet the females were bred and kept throughout pregnancy. Under such dietary conditions few animals carry their young to term. Many females become sterile; some stop having oestrous cycles; others mate but resorb their young. In 1 series of experiments (Warkany and Schraffenberger, '46) only 7 of 140 females carried their offspring to term and 39 young were obtained. All the eyes of the young that were serially sectioned were found to be abnormal. The most constant abnormal finding was a fibrous retrolenticular membrane in place of the vitreous body (fig. 1B-H). In addition there were frequently colobomas, eversion, abnormal structure and folding of the retina, rudimentary development of the iris and of the ocular chambers, defects of the cornea and of the conjunctival sac, and lack of fusion of the lids. In the last case the abnormal eyes could be recognized by external inspection ("open eyes") (fig. 2). These experiments on rats were repeated with slight modifications by Jackson and Kinsey ('46) who confirmed the findings of Warkany and Schraffenberger. The study of Jackson and Kinsey was supplemented by determinations of the vitamin A level of pregnant rats which was found to be less than 12 I.U. per 100 ml in all the mothers who produced young with abnormal eyes. In the series of these authors only 4 of 57 females subjected to the dietary conditions described carried their fetuses to advanced stages of development, and Jackson and Kinsey concluded "that the ocular defects occur in the young rat only when the maternal vitamin A deficiency is extremely severe, so ad-

vanced, in fact, that fetal resorption is common and normal birth is impossible.”

Since the rate of fertility was very low in the original experiments and the yield of abnormal offspring very small, improvements of the experimental methods were attempted by modifications of the preparatory diet. These modifications are reported in the present communication. In the previous experiments rats of the Sprague-Dawley strain only were used. It seemed advisable to repeat the experiments with rats from a different source and in this series a large number of rats obtained from the Albino Farms, Red Bank, N. J. were included.

#### METHODS

Female rats weighing 30 to 40 gm were placed on the preparatory diet (diet U) which had the following percentage composition: Ground whole wheat, 74; crude casein,<sup>2</sup> 15; brewers' yeast,<sup>3</sup> 10; sodium chloride of C.P. grade, 1. According to the supplements added to this diet the animals were divided into 4 groups. One hundred and sixty-five females were given a supplement of only 4  $\mu$ g of carotene every tenth day (group I). Since this supplement proved insufficient for growth and maturation, each rat received in addition approximately 2 gm of frozen horsemeat daily. This group was kept on diet U until mating occurred, at which time the females weighed between 150 and 160 gm. Then the females were placed on diet W. Group II, consisting of 34 females, received a supplement of 12  $\mu$ g carotene every tenth day. In group III 33 animals were given 25  $\mu$ g carotene every tenth day. A fourth group consisting of 28 females received during the first month supplements of 25  $\mu$ g of carotene every tenth day. In the following month a loss of weight was observed and therefore the carotene intake was doubled during the last 2½ weeks of the preparatory period. Groups II, III and IV were placed on diet W upon reaching maturity and attempts to breed them were made after the change to this purified diet. In these 3 groups mating took place usually within 1 week or 10 days after the

<sup>2</sup> Borden.

<sup>3</sup> Mead Johnson.

change to diet W; but even after 4 weeks on the purified diet mating occasionally occurred. Diet W, a purified diet completely free of vitamin A and carotene, had the following percentage composition: Sucrose, 68; vitamin test casein, 18; vegetable oil, 10; salt mixture of Hubbell, Mendel and Wake-man ('37), 4. To 100 gm of this diet was added 0.8  $\mu$ g thiamine hydrochloride; 0.8  $\mu$ g pyridoxine hydrochloride; 0.8  $\mu$ g riboflavin; 1  $\mu$ g calcium pantothenate; 10  $\mu$ g niacinamide; and 100  $\mu$ g choline chloride. This diet was supplemented every tenth day by vitamin D (1 drop of a 1:4 dilution of drisdol with olive oil), vitamin E (1 drop of a solution of 5 gm alphatocopherol in 100 ml of olive oil), and vitamin K (1 drop of a solution of 1  $\mu$ g of 2-methylnaphthoquinone in 5 ml of olive oil). On this diet the female rats were bred and kept throughout pregnancy. Vaginal smears were made daily and the weight curves were closely observed. This made possible the recognition of the phase of oestrus; the beginning of pregnancy (by finding sperm); the continuation of pregnancy; or beginning of resorption (by finding of large amounts of blood in the vaginal smear and by a marked loss of weight). The day on which sperm were found was counted as the first day of pregnancy. The profuse bleeding of resorption can be distinguished as a rule from the normal blood sign, which appears between the tenth and the fifteenth days of gestation and which consists of a small amount of blood in the vaginal smear. When early termination of pregnancy threatened, the animal was opened and the fetuses removed. When the young were carried to term the mothers were opened on the twenty-second day of gestation. Many young thus removed were alive but none could be raised. The mothers were sacrificed and therefore no second litters were obtained. The eyes of at least 1 animal in each litter were sectioned serially. If no retrolenticular membrane was found the animal was considered normal but if a membrane was seen in any section the animal was considered abnormal. When the eyes of 1 young in a litter were found to be abnormal the entire litter was considered and counted as abnormal. This method of classification appeared

to be justified, since in 14 litters selected at random the eyes of all the littermates when serially sectioned were found to be so similar that all the members of each litter could be considered normal or abnormal (fig. 1). There was also marked external resemblance between the animals of the same litters (fig. 2).

#### RESULTS

Of 260 females reared on the preparatory diet U, 22 had cycles but did not mate. One hundred and twenty-four females mated but did not have any issue. Litters were obtained from 114 females but only 30 of these litters were carried to term (table 1). The rest were obtained prematurely and consisted of fetuses of various gestational ages (table 2). Of the females used in this series 156 were of the Sprague-Dawley strain and 104 were obtained from the Albino Farms.

According to the presence or absence of a retrolenticular membrane in the eyes of 1 or of several members of a litter, 89 litters were considered abnormal and 25 normal. These litters consisted of a total of 820 young, 612 of which were considered abnormal and 208 normal. In addition to the retrolenticular membrane, other ocular defects resembling those described in our previous publication ('46) were seen in many of the eyes sectioned. In most eyes with a retrolenticular membrane the vitreous humour is absent and fibrous tissue occupies the entire space between the lens and the retina. However, in some of the abnormal eyes rudiments of the vitreous body are present and are transversed by a thick strand of fibrous tissue which starts at the head of the optic nerve and continues to the posterior surface of the lens (fig. 1). Many litters show external changes which are not observed in control animals. In figure 2 an abnormal litter of 7 young is compared to a normal newborn animal. The abnormalities are smaller than the normal animal although they were removed from the mother's uterus on the twenty-second day of gestation. Owing to the edematous swelling of the subcutaneous tissue the narrowing at the neck is markedly reduced and the entire back presents a single convexity. All the animals of



this litter have "open eyes" which are best seen in specimens B and D. Subcutaneous hemorrhages are seen in C and D. The fingers and toes are well-developed but the dorsa of the

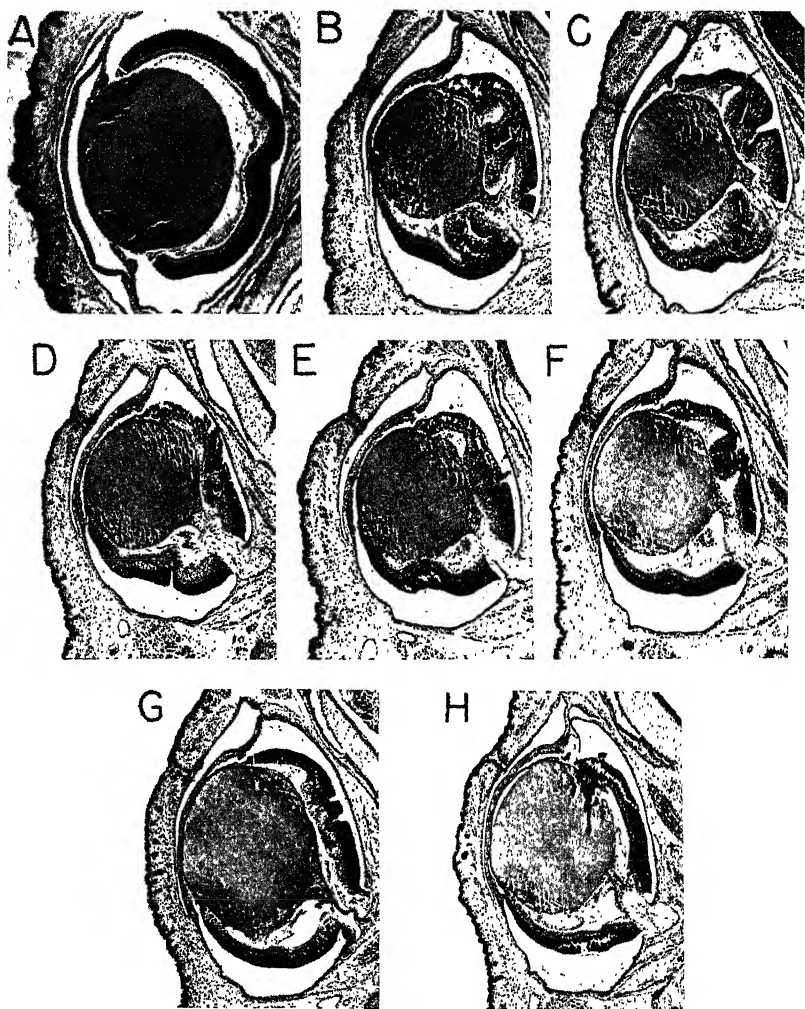


Fig. 1 Sections through eyes of newborn rats. A, control; B-H, offspring of vitamin A deficient mother. These sections represent the eyes of 7 littermates. The retrolenticular membrane is seen in every section, while only rudiments of a corpus vitreum are developed. A slight eversion of the retina is seen in section G only. ( $\times 18$ ).

hands and feet are puffy. The soles of the feet are turned inward which represents a retention of the fetal position.

In many young with abnormal eyes serial sections of the trunk and abdomen revealed various congenital anomalies of



Fig. 2 A, newborn rat, control; B-H, offspring of vitamin A deficient mother, removed from the uterus on the twenty-second day of gestation. These young, all littermates, show edema and inward rotation of the feet. The "open eye" can be seen in B and D, a cutaneous hemorrhage in D.

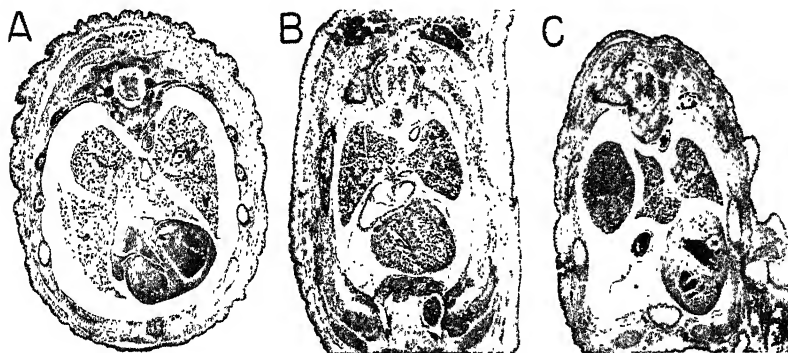


Fig. 3 Sections through the chest of newborn rats. A, control ( $\times 3\frac{1}{2}$ ); B and C ( $\times 5\frac{1}{2}$ ), offspring of vitamin A deficient mothers. In B the lung, the pleura and the heart muscle show retarded development. In C a lobe of the liver is seen in the right pleural cavity (diaphragmatic hernia).

the soft tissues. The lungs are underdeveloped and frequently retain their fetal position behind the heart and fail to grow forward. The pleural chambers border the pericardial cavity posteriorly and laterally but do not surround it (fig. 3 B and C). The heart muscle retains the spongy structure of the fetal

heart (fig. 3 B and C). In a number of specimens the right dorsolateral portion of the diaphragm is not developed and a lobe of the liver may protrude into the pleural space. This represents a diaphragmatic hernia (fig. 3 C). The kidneys are hypoplastic and the renal pelvis and the ureters are not distended as in the normal rat at the end of the gestational period. The space between the kidneys is narrow and fusion may take place in the lower poles, thus forming a horseshoe kidney. The testes are frequently undescended and may be found at the level of the kidneys or above (fig. 4 C). A detailed study

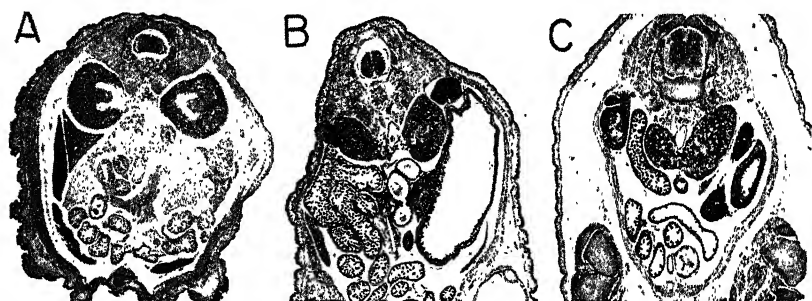


Fig. 4 Sections through the abdomen. A, control ( $\times 3\frac{1}{2}$ ); B and C, offspring of vitamin A deficient mothers. In B the renal pelvis seen in the left kidney is not unfolded ( $\times 5$ ). In C (20-day fetus;  $\times 7$ ) a fusion of the lower poles of the kidneys is seen (horseshoe kidney).

of these anomalies is being made at the present time and will be reported elsewhere. There is a tendency for hemorrhages to occur in the skin, the eyes and other organs. Keratinizing metaplasia was found by Wilson and Warkany ('47) in many abnormal young in the urethra distal to the termination of the genital ducts. In other parts of the body no abnormal keratinization was observed.

In all 4 groups abnormalities of the same type were obtained and there was no difference between the abnormalities in the offspring of rats of the Sprague-Dawley strain and those obtained from the Albino Farms. However, there were differences in the incidence of abnormalities and in the fer-

tility rates in the 4 groups according to the different amounts of carotene in the preparatory period.

Groups I and II had the lowest fertility rate and the highest incidence of abnormal young; group IV had the highest fertility rate and the lowest incidence of abnormal young.

Table 2 shows that most of the younger fetuses that were dying in utero or were threatened by intrauterine death were

TABLE 1

*Breeding results with females raised on the preparatory diet U with various supplements and bred on diet W.*

CATEGORY OF INTEREST	TOTAL	SUPPLEMENTS OF PREPARATORY DIET			
		Group I: 4 $\mu$ g carotene <sup>1</sup> (and horsemeat)	Group II: 12 $\mu$ g carotene <sup>1</sup>	Group III: 25 $\mu$ g carotene <sup>1</sup>	Group IV: 25-50 $\mu$ g carotene <sup>1</sup>
Number of females	260	165	34	33	28
Cycles but no matings	22	17	3	2	0
Matings but no issue	124	88	19	13	4
Number of litters	114	60	12	18	24
abnormal	89	52	12	12	13
normal	25	8	0	6	11
Number of young	820	428	76	139	177
abnormal <sup>2</sup>	612	363	76	96	77
normal <sup>2</sup>	208	65	0	43	100

<sup>1</sup> The carotene supplements were given every tenth day. The animals in group I received 2 gm frozen horsemeat daily.

<sup>2</sup> These figures are based on the assumption that the entire litter was abnormal, when anomalies were found in the eyes of at least 1 member of the litter (see page 4).

TABLE 2

*Gestational age of 114 litters and of 820 young.*

DAY OF PREGNANCY	15th	16th	17th	18th	19th	20th	21st	22nd
Total number of litters	3	6	8	7	10	15	35	30
abnormal	3	6	7	7	9	15	25	17
normal	0	0	1	0	1	0	10	13
Total number young	14	28	47	24	79	105	272	251

abnormal, while about one-third of the survivors of the gestation period were normal.

Eighty females received in the same shipments as the experimental animals were used as controls. They were fed an adequate stock diet throughout their growing and breeding periods and mated to the same males as the females on the deficient diets. They delivered a total of 104 litters and 822 young. The eyes of 60 of the young, each representing a different litter, were serially sectioned. No retrolental membrane was found in any of the eyes examined. Sixty control offspring were dissected and neither diaphragmatic hernias nor horseshoe kidneys were found. The kidneys of 33 control offspring were found to be normal in serial sections.

#### DISCUSSION

The experiments reported here represent an attempt to produce congenital malformations in the offspring of rats by maternal vitamin A deficiency. We intended to breed the females in a borderline state of vitamin A deficiency which would alter the development of the offspring without causing intrauterine death of the embryos. As this aim could be realized only in part, because sterility and intrauterine death on one side and normal development of the young on the other side interfered with the experiment, we attempted to regulate some of the factors which improve the yield of abnormal offspring. In our previous communication ('46) only 36 litters were obtained from 140 females. This corresponds to a fertility rate of 25.7%. In the present series the fertility rate was higher, since 114 of 260 females or 43.8% had litters. The increase in fertility rate resulted, however, in the birth of some normal offspring. It was shown in the experiments reported here that the character of the preparatory diet influenced the results to a certain extent. In general, one can expect that improvement of the dietary conditions will result in an increased fertility rate and in a lower percentage of abnormal offspring. While abnormalities of the same type can be obtained in the offspring of mothers who receive any

of the preparatory diets employed, it seems advisable to supplement diet U with 12-25  $\mu$ g carotene every tenth day.

#### SUMMARY

Congenital abnormalities can be induced in the offspring of female rats reared and bred on diets deficient in vitamin A. The abnormalities are found in soft tissues and are different from those induced in the skeleton by maternal riboflavin deficiency.

Variations of the preparatory diet influence the yield of abnormal young. Increase of the carotene supplement results in an increased fertility rate, but the percentage of abnormal young decreases. Reduction of the carotene supplements reduces fertility while the percentage of abnormal young increases.

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# THE NUTRITION OF THE MOUSE

## II. EFFECT OF DIET ON THE BACTERIAL FLORA OF THE INTESTINE AND THE CECUM <sup>1</sup>

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It is well-known that diet influences the intestinal flora of several species of animals, as is shown by the work of Tissier ('08) and later Sanborn ('31) with humans, Belonovsky ('07) with mice, Herter and Kendall ('09-'10) with cats and monkeys, Hull and Rettger ('15, '17) and Porter and Rettger ('40) with rats, and Crecelius and Rettger ('43) with guinea pigs. In general, all of the species responded similarly whenever either of 2 types of diets was fed. Meat or a high protein diet produced proteolytic flora consisting largely of coliform organisms; a variation in the carbohydrate induced a change toward a fermentative flora. For example, milk or lactose, and sometimes dextrin, encouraged an aciduric bacterial population; when dextrin was fed, there was produced a type of flora which synthesized some of the B vitamins (Guerrant et al., '35; Schweigert et al., '45).

Belonovsky ('07) studied only the effect of lactose and milk on the bacteria of the feces and upon the ease of implantation of certain strains of bacteria in the intestine. Little attention was paid to the effect of varying the diet on the predominating

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flora or the effect that such a change would have in the nutrition of the mouse.

It has been shown in this laboratory that considerable difference exists in the nutritional requirements of several highly inbred strains of mice. In general, the requirements of the C<sub>57</sub> strain (black, with low incidence of spontaneous mammary tumors) are lower than those for the A strain (albino, highly susceptible to spontaneous mammary tumors). Both of these strains have been used in these studies of the intestinal flora, but this paper deals principally with the A strain.

The studies to be reported here were undertaken to determine the effect of 2 synthetic rations and a natural diet upon the predominating bacteria in the intestinal tract of A strain mice, and whether any such change could affect the nutrition of the host. The lower parts of the small intestine and the cecum were chosen for investigation since it was felt that these were probably the most likely sites of absorption by the mouse of any synthetic products liberated by the bacteria.

The predominating bacteria of these segments of the intestine were studied by Gram stains and by culturing serial dilutions of the contents. Direct bacterial counts were made on the samples and the entire cecal contents were weighed.

#### EXPERIMENTAL

Weanling mice of the A strain, 21 days old, were placed in individual screen-bottom cages, and food and water supplied *ad libitum*. Growth rates were measured by weighing the animals periodically. The stock ration<sup>2</sup> and 2 synthetic diets (nos. 101 and 133) were used. The composition of the synthetic rations has been described in detail elsewhere (Fenton and Cowgill, '47). Diet 101 contained 23% casein and 60% dextrose, while diet 133 contained 30% casein and 45% dextrin. In other respects the 2 diets were similar except that no. 133 also had cystine, vitamin K, biotin and folic acid added.

Representative animals from these 3 groups were killed by etherization and were opened immediately using sterile tech-

<sup>2</sup> Purina Laboratory Chow.

nique. The cecum and the lower half of the small intestine were removed and the contents from these segments squeezed directly into sterile, tared vials which were then plugged with cotton. The upper part of the small intestine was not studied because of the small numbers of organisms present. The large intestine was not considered to be a likely area of absorption of bacterial excretory products because on the synthetic diets this material formed into hard pellets immediately upon leaving the cecum. It seemed likely that any substances that might be produced by bacteria in this region would be lost to the host. The vials containing the samples were weighed and the samples made up to a known dilution with sterile distilled water. The vials containing the diluted samples (usually a 1:25 to 1:100 dilution, depending on the size of the sample) were stoppered and shaken thoroughly. Any clumps of contents were broken up with a sterile pipette. The samples were then further diluted to  $10^{-4}$ , and then serially by steps of 100 until a dilution of  $10^{-10}$  was reached.

The cultural work was done first, and the whole procedure after the removal of the contents from the body was done as quickly as possible in order to keep the sample from being exposed unduly to air and low temperatures. The cecal contents were cultured first, as the bacteria isolated from this segment seemed to be more anaerobic than those from the small intestine. Attempts to reduce dissolved air to a minimum consisted of plugging the water blanks with rubber stoppers immediately upon removal from the autoclave, and of boiling the broth for 10 minutes in a water bath, followed by immediate cooling in water just before inoculation. The inoculum was placed carefully under the surface of the broth with a pipette, and the tube was sealed with vaspar.<sup>3</sup> The broth was composed of 1% of each of the following: Bacto-peptone, tryptone, peptonized milk, yeast extract, beef extract, and glucose, and 0.5% of  $K_2HPO_4$  all dissolved in liver extract made by extracting 75 gm of Bacto liver powder in a

<sup>3</sup> Composed of equal weights of petrolatum and paraffin.

liter of distilled water. The pH was adjusted to 6.8 before autoclaving.

For the cecal cultures, inocula representing  $10^{-8}$ ,  $10^{-9}$ , and  $10^{-10}$  were put into the broth in duplicate while for the small intestine, only dilutions  $10^{-8}$  and  $10^{-9}$  were cultured. The cultures were incubated for 1 week at  $37^{\circ}\text{C}$ . Only these higher dilutions were cultured, as this study was primarily concerned with the predominating organisms. However, in order to learn more about the distribution of coliform organisms, in most cases the E C medium of Hajna and Perry ('43) was inoculated with dilutions of the samples from  $10^{-2}$  to  $10^{-7}$ .

As soon as growth appeared in any tube, the culture was Gram stained, the motility was determined, the pH of the broth was measured electrometrically, and agar shake cultures were made to indicate the degree of anaerobiosis and to permit purification if necessary. The agar shakes were saved so that certain synthetic activities of these cultures could be studied. The results of this phase of the work will be presented in the following paper (Gall et al., '48).

Immediately after the completion of the cultural procedure, a Gram stain was made of the diluted material from the original vial. From this the predominating morphological types and their approximate relation to each other were noted. Direct counts were also made on the sample after suitable dilution. In general the cecal content was diluted 1:1000, while the small intestinal content was diluted 1:25 to 1:100, depending on the expected bacterial count. The slide counts were made on  $2 \times 2$  cm areas by mixing 0.01 ml of the diluted sample with 1 drop of saturated nigrosine solution in methyl alcohol spread evenly over the area. The slides were dried very quickly on a hot plate, and the counts were made with a microscope for which the field had been calibrated. It was easy to see the unstained bacteria against the black background, and from the average of 10 fields on each of 2 slides (about 300-400 organisms), it was possible to calculate the bacterial count per gram of original sample.

The weight of the cecal and small intestinal contents was determined to the nearest milligram on an analytical balance. The material came out of the cecum very easily, and it is felt that the weights obtained represented with fair accuracy the entire cecal contents. The weights of the contents of the small intestines were extremely variable, since the position of the lumps of food in the intestine was so irregular that the inclusion or exclusion of 1 mass of food would alter the result severalfold. Therefore not much significance is attached to these figures, and they are not reported in the table.

In addition to these main experiments on A strain mice a few preliminary studies were carried out on small groups of C<sub>57</sub> and A strain mice on various other diets under investigation in the laboratory.

#### RESULTS

Growth rates of male mice fed the stock ration or diet 101 have been reported by Fenton et al. ('47). All 3 diets studied here supported growth about equally well. Fenton and Cowgill ('47) have reported complete failure in securing reproduction and lactation in mice raised and maintained on ration 101. The more complete diet 133 did support reproduction and lactation, but the results were still not as satisfactory as with the stock ration.

Gram stains of the cecal contents of the animals fed these 3 diets were examined microscopically, and the differences in the flora on these diets were sufficiently marked to permit identification of the diet fed, simply by studying the slide. The cecal contents of the animals on stock ration were characterized by the presence of a slender, gram-negative, curved rod and a cigar-shaped organism. Except for some gram-positive rods, probably lactobacilli, there were few other bacteria present. In contrast to this simple flora, the animals on diet 101 harbored a varied bacterial population, but there was always present in these animals a considerable number of large round cocci, occurring in pairs and short chains. This coccus was characteristic of this diet. Diet 133 also supported

a variety of organisms, but the flora differed from that on ration 101 in several ways. The large coccus found on diet 101 was absent, but in its place was found in smaller numbers an elongated coccus, occurring in pairs, and a tiny coccus forming a characteristic, closely united chain of 10-20 organisms. On diet 133 there also appeared in large numbers, a highly curved rod that formed a perfect C.

The Gram stains of the small intestinal contents from animals fed all 3 diets showed the gram-positive rod as the predominating bacterium, but on the 2 synthetic diets the coccus characteristic of that ration usually appeared.

A more complete description of the types of bacteria found on these various diets, as well as some notes on their synthetic activities, especially in regard to B vitamins, will be presented in the following publication.

As shown in table 1 the cultural differences among the 3 groups were also marked and largely confirmed the conclusions drawn from the examination of the slides. The cecal samples cultured from stock diet animals gave scanty growth, due to the predominance of the slender curved rod, an anaerobe which proved very difficult to culture. In no case did the cultures from the animals on this diet grow in as high a dilution as was expected from the slide count. Virtually the only organism isolated from these animals was the easily cultivatable gram-positive rod.

In contrast, the bacteria from the ceca of animals fed diet 101 grew very readily. In 9 out of 12 cases the cultures grew in the dilutions indicated by the slide counts. The characteristic coccus was easily isolated, as were the gram-positive rods, and the coliform organisms when the latter were present. Gas, usually found in 1 or more tubes in the series, could in some instances be attributed to coliforms. However, in some tubes only a slight amount of gas appeared. On staining these cultures only cocci were observed, and attempts to isolate coliforms from these tubes repeatedly failed. This cultural picture identified the animals which had been fed diet 101.

TABLE 1  
Summary of the bacterial counts, weights of cecal contents and cultural results obtained from *A strain micc.*

DIET NUMBER	101			133			STOCK		
	Small 12	Cecum 12	Small 12	Cecum 12	Small 9	Cecum 9	Small 9	Cecum 9	Cecum 9
No. of animals showing agreement of slide and cultural "counts",	12	9	10	3	9	0			
Wt. of segment contents, gm	1	0.065 ± 0.021 <sup>2</sup>	1	0.094 ± 0.029 <sup>2</sup>	1	0.407 ± 0.033 <sup>2</sup>			
Bacterial count—									
Billions per gm of sample									
per intestinal segment	0.710 ± 0.81 <sup>2</sup>	22.2 ± 11.5 <sup>2</sup>	0.321 ± 0.26 <sup>2</sup>	23.0 ± 11.8 <sup>2</sup>	0.875 ± 0.99 <sup>2</sup>	42.8 ± 11.5 <sup>2</sup>			
No. of cultures isolated; according to type	1	1.5 ± 0.84 <sup>2</sup>	1	2.09 ± 1.15 <sup>2</sup>	1	17.9 ± 5.4 <sup>2</sup>			
Gram + rods									
10 <sup>-5</sup>	14	7	9	10	13	13			
10 <sup>-6</sup>	7	6	3	5	8	8			
10 <sup>-8</sup>	1	3	1	0	1	0			
Round coccus									
10 <sup>-5</sup>	7	15	0	0	0	1			
10 <sup>-6</sup>	3	9	0	0	0	0			
10 <sup>-8</sup>	1	5	1	0	1	0			
Elongated coccus									
10 <sup>-5</sup>	0	0	1	6	0	0			
10 <sup>-6</sup>	0	0	0	3	0	0			
10 <sup>-8</sup>	1	0	1	0	1	0			
Tiny coccus									
10 <sup>-5</sup>	0	0	1	3	0	0			
10 <sup>-6</sup>	0	1	0	4	0	0			
10 <sup>-8</sup>	1	1	1	1	1	0			
Curved rod									
10 <sup>-5</sup>	1	0	1	1	0	1			
10 <sup>-6</sup>	0	2	0	6	0	1			
10 <sup>-8</sup>	1	3	1	1	1	0			
Coliforms									
10 <sup>-5</sup>	0	4	0	3	0	0			
10 <sup>-6</sup>	0	3	0	0	0	1			
10 <sup>-8</sup>	1	3	1	0	1	0			
No. of animals showing coliform organisms 100 M or above	1	5	1	2	1	1			

The cecal contents from animals fed ration 133 gave cultural results somewhat different from the other 2 diets. In only 3 cases out of 12 did the cultures grow in the dilution expected from the slide counts, but the discrepancy was not as great as with the stock ration. The elongated coccus and the tiny coccus, which proved to be an anaerobe, occurred in a fair number of cultures. The gram-positive rods were also isolated regularly. The curved, C-shaped rod seen on the smears was never isolated in that morphological form.

It has commonly been thought that coliform bacteria are always among the predominating organisms in the intestinal tract, but this study indicates that with A strain mice its occurrence in large numbers varies with the diet. With the average bacterial count in the neighborhood of 22 billion per gram of cecal contents, coliforms were isolated in dilutions of 1 billion or more only 7 times. Three of these came from the 10 billion dilution and 4 from the 1 billion. These cultures represented 5 animals, 4 of which were fed diet 101. Therefore, in only 10% of the animals tested were coliforms present in the top 2 dilutions, and in only 20% of the cases in the top 3 dilutions. It is pertinent to point out that 41% of the animals on diet 101 had coliforms present in 100 million or more, as compared to much fewer in animals on the other 2 diets. Two-thirds of the animals tested in low dilutions showed less than 1 million coliform organisms per gram of cecal contents and at least 25% were below 10 thousand per gram. It is of interest to note that with the stock ration animals where the opportunity for coprophagy was the greatest, there was the mildest coliform flora.

The cultural results of the small intestinal contents on the 3 diets reflected the findings with the Gram stain. All 3 diets gave a large number of cultures of gram-positive rods, while the 2 synthetic diets showed in addition cocci of the type characteristic of the ration.

The 3 diets were compared as to bacterial count per gram of cecal material and total bacterial count per cecum. For reasons stated above, a similar study for the small intestinal

contents is not reported in table 1. The animals eating the stock ration showed a higher bacterial content when measured per gram or per cecum, although the difference is greater by far when measured by the latter criterion. This is a function of the greater content of the cecum of the animals eating the natural diet. By statistical analysis these differences were found to be highly significant ( $p < 0.01$ ). There was little difference between the animals eating the 2 synthetic diets, however. Statistically there was no difference between the cecal count per gram on diets 101 and 133. A comparison of the total cecal counts of these diets also showed no significant difference ( $p = 0.2$ ).

The cecal contents obtained from animals fed the stock ration were statistically highly significantly heavier than the contents from the animals on the 2 synthetic diets ( $p < 0.01$ ). The animals on diet 133 showed greater cecal contents than those on 101 ( $p$  just equals 0.01). This should be interpreted cautiously, however, for reasons to be discussed below.

By way of preliminary investigation a few animals of both  $C_{57}$  and A strains were studied after having been maintained on the following diets: (a) 3 diets similar to no. 133, but with dextrose as the carbohydrate, and a diet identical with no. 101 (containing dextrose), but omitting roughage; (b) a diet low in riboflavin and one low in pantothenate, but in other respects similar to no. 101; (c) a diet similar to no. 101 with 1% sulfathalidine added; (d) a ration identical with no. 101, but replacing the dextrose with dextrin.

The diets listed under (a) showed a cecal flora essentially the same as that found on ration 101. The animals deficient in riboflavin and pantothenate showed the same cecal flora as those on diet 101 except that a C-shaped rod was also present. The mice fed sulfathalidine showed a flora similar to those on ration 101, but the weights of their cecal contents were somewhat greater. When the dextrose of diet 101 was replaced with dextrin, the resulting flora resembled that which was found on the dextrin containing diet no. 133. In all of these preliminary studies it must be emphasized that only a micro-



scopic examination was made, thus affording a comparison on the basis of morphology alone.

A group of mice was fasted 24 hours and then fed the stock ration. Small numbers of these animals were sacrificed and their cecal contents studied at various intervals following feeding. The animals sacrificed 3.5 to 6 hours after feeding showed a C-shaped rod, which was not present in animals permitted to feed until sacrificed or in mice sacrificed up to 2.5 hours after feeding, following a period of fasting.

#### DISCUSSION

Ample evidence has been presented in the main portion of this study and also in the preliminary work reported here showing that the composition of the diet can have a marked effect upon the intestinal flora of the experimental animals. The bacteria seen on each of the 3 rations investigated have been so distinctive that it was possible to judge by microscopic examination alone which diet had been fed to any particular animal. In fact, following the initial experiments, we made it a practice to give the investigator (L.S.G.) an animal for the day's work without her having any knowledge of the nature of the ration. On the basis of the Gram stain, the examiner would attempt to identify the diet fed, and in only 1 case out of 33 was an error made.

Many investigators have implied that a great deal of the intestinal synthesis should be ascribed to coliform bacteria. Our data do not warrant such conclusions, at least for this highly inbred strain of mouse. We have found appreciable numbers of coliforms only on diet 101, and here only 5 out of 12 animals showed this organism in the top dilutions.

It is important to stress that with the animals fed the stock ration and diet 133, we frequently failed to obtain cultural results commensurate with the slide count. More work is necessary to determine the cultural requirements of the bacteria which predominate on this diet.

In view of the greater roughage content of the stock ration, it was to be expected that animals on this diet would have

greater cecal contents than the animals on synthetic diets. In our studies comparing 2 synthetic diets, 1 containing dextrin (no. 133) and the other dextrose (no. 101), there was a statistically significant difference between the weights of the cecal contents of the 2 groups ( $p$  just equals 0.01). However it must be stated that the animals fed diet 133 were slightly older than those eating no. 101, and for this reason the results should be interpreted with caution. Mannering et al. ('44) found that rats fed a synthetic diet containing dextrin had a larger cecal content per unit of body weight than animals fed a similar diet containing sucrose.

It must also be stated in this connection that 6 of the 9 mice fed the stock ration were older than the animals on synthetic ration, while 3 were the same age or younger. On comparison the average weights of the cecal contents of the young and old animals on stock ration were found to be so nearly alike ( $0.386 \pm .038$  and  $0.417 \pm .045$ , respectively), that it was felt that these age differences did not seriously affect the interpretation.

The study reported here on both  $C_{57}$  and A strains of mice on different diets gives evidence that there is a flora, characteristic of mice fed a dextrose diet, which is different from that of animals maintained on a ration containing dextrin. This is in agreement with the findings of other workers on several species cited in the introduction, that certain carbohydrates induce differences in intestinal flora.

#### SUMMARY AND CONCLUSIONS

1. Microscopic and cultural examinations of the small intestine and the cecum of A strain mice on stock ration and 2 synthetic diets were carried out.

2. Mice fed each of these diets had a characteristic cecal flora; on the stock ration it consisted of a slender, gram-negative, curved rod; on the 2 synthetic diets cocci characterized each of them.

3. Coliforms were not found to be present in significant numbers on 2 of the diets and were found in large numbers in less than half of the mice on diet 101.

4. The mice on the stock diet had a larger bacterial population per gram of cecal contents and per cecum than the mice on synthetic diets. There was no significant difference between the 2 groups of mice on synthetic diets.

5. The weights of the cecal contents of the animals on stock ration were greater than those of the animals fed synthetic diets. There was little difference between the animals on synthetic diets with respect to weights of cecal contents.

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# THE NUTRITION OF THE MOUSE

## III. RELATION OF DIET TO THE SYNTHETIC ACTIVITY OF THE PREDOMINATING FLORA ISOLATED FROM THE SMALL INTESTINE AND CECUM <sup>1</sup>

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It has been established that the bacteria of the intestinal tract are active in the synthesis of certain B vitamins (Mitchell and Isbell, '42), and it has been demonstrated that the cecum is probably the site of this synthesis (Taylor et al., '42; Schweigert et al., '45). It has also been shown that certain carbohydrates such as dextrin promote greater intestinal synthesis of these B vitamins than the simpler sugars, sucrose and dextrose (Guerrant et al., '35; Schweigert et al., '45).

In most of these studies, the synthetic activities of the whole intestinal flora as found *in vivo* have been investigated, with little attempt to isolate and study the synthetic activities of the predominating flora. Also since it is the vitamin content of the fecal flora that has been the subject of several studies, it has not been clearly demonstrated whether the vitamins are available to the host, unless coprophagy is practiced or considerable autolysis occurs in the intestinal tract.

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The purpose of our preliminary studies has been to determine whether any of the predominating organisms isolated from the small intestines and ceca of mice fed different diets showed any marked differences in their synthetic activities with respect to the B vitamins, especially the occurrence of the vitamins in the medium surrounding the bacterial cells. We have observed in this laboratory (Fenton and Cowgill, '47a, b) that mice of the C<sub>57</sub> and A strains differed in their requirements for riboflavin and pantothenic acid. It seemed to us that possibly this difference could be attributed to differences in the rates of intestinal synthesis.

#### EXPERIMENTAL

The methods for maintenance of the mice and for isolation of the predominating flora have been presented in the preceding paper (Gall et al., '48). A description of the diets or a reference to their composition will also be found in that paper.

The cultures were obtained from 62 mice of the A and C<sub>57</sub> strains fed 7 different diets, which can be grouped into 3 main classes: a stock diet and 2 synthetic diets, 1 containing dextrin and the other dextrose. This study included the testing of a total of 107 cultures, of which 83 came from the 3 top dilutions of the cecal contents, 18 from the 2 highest dilutions of the small intestinal contents, and 6 were coliform organisms from lower dilutions of contents from both intestinal segments.

The organisms were tested within 1 week of isolation in most cases. Cultures for testing were obtained by planting a single colony from an agar shake culture prepared from the original broth into a complete synthetic broth (Krehl and Illingworth), modified by using the casein hydrolysate described by Roberts and Snell ('46). Since most of the original cultures showed only 1 type of bacterium in the Gram stain, little difficulty was experienced in obtaining a pure culture. In those few cases where a tested organism came from a mixed culture, a series of agar shake cultures was made to insure a well-isolated colony. To check for purity, Gram stains

were made from the broth planted with the single colony, and from the culture representing the third transfer in complete broth.

After the culture in complete broth had been incubated for 24 hours at 37°C., it was observed for growth. If turbidity was apparent, 0.1 ml of the culture was used as an inoculum for the various broths employed to test its synthetic abilities. The media inoculated were as follows: a complete synthetic broth of the same composition as that in which the organism had been growing, and 5 deficient broths, identical with the complete broth, except for the omission of 1 of the following vitamins from each broth — riboflavin, niacin, biotin, folic acid and pantothenic acid.

All broths for the series of 3 transfers and the subsequent microbiological assays on these cultures were prepared simultaneously once each week. Three serial transfers were made of each culture at 24-hour intervals, each culture being transferred into a broth identical with the one on which it was growing. Immediately prior to the inoculation with 0.1 ml of the culture, the broths were autoclaved and cooled rapidly. All anaerobic cultures were sealed with vaspar. The growth of the cultures was graded from 1 + to 4 +, indicating the degree of turbidity produced. If a tube failed to show visible growth before the third transfer, it was carried for one more transfer before being discarded.

A slightly different procedure was used for the coliforms after it became apparent that the synthetic broth referred to above would not support satisfactory growth of the coliforms. A mineral salt and glucose broth (MacLeod, '40) was used for the serial transfers.

If the culture survived 3 transfers in any of the deficient broths, the supernatant fluid obtained by centrifuging the 24-hour culture was tested for the presence of the omitted vitamin. This was done by microbiological assay, following the procedure of Snell ('47) except for a modification of the broth. The synthetic medium used in this study was the broth of Krehl and Illingworth (unpublished data), modified as



noted above. The assay was carried out on 1, 2 and 4 ml of the supernatant broth, using *L. casei* as the test organism for riboflavin and folic acid, and *L. arabinosis* for niacin, biotin and pantothenic acid. No regular standard curve was run — only a blank and a maximum tube — as it was not desired to estimate the amount of B vitamin quantitatively, but merely to determine whether large or small amounts of the vitamin were liberated from the cells. The results were graded on a scale running from 1 + to 4 +. The 1 + and 2 + values were interpreted as indicating slight synthesis and liberation of the vitamin, while 3 + and 4 + values meant that the titration approached or equalled that of the maximum tube, indicating considerable amounts of the vitamin to be present in the broth. A value was not considered significant unless the titration of the tube containing 1 ml of the supernatant fluid was greater than the blank, and unless the titration increased as the amount of the added supernatant fluid increased.

### RESULTS

In general the predominating organisms isolated from the intestinal contents of the mice on the different diets were of 7 types. A table showing the distribution of 6 of these types isolated from A strain animals on different diets will be found in the preceding paper (Gall et al., '48). The seventh type occurred mostly in 10 out of 12 black mice on diet 101, and will be discussed here. The distribution of the other organisms from the C<sub>57</sub> mice was about the same as that for the white mice on the same diets.

A summary of a few characteristics of the bacteria isolated is presented in table 1. None of the organisms isolated was motile or formed spores, so these characteristics were not included in the table. No attempt has been made to key out these bacteria, but some tentative genus names will be suggested for 3 of the types of organisms. The gram-positive rods were probably *Lactobacilli*, and the gram-negative anaerobic rods, both the curved and the short fat ones, resembled certain of the *Bacteroides* as described by Eggerth and Gag-

TABLE 1

*Some characteristics of 7 types of bacteria isolated from high dilutions of small intestinal and cecal contents of mice on several different diets.*

ORGANISM TYPE AS DESIGNATED IN TEXT	MORPHOLOGY	GRAM REACTION	OXYGEN RELATION	GROWTH IN ORGANIC BROTH	FINAL pH IN ORGANIC BROTH	GAS PRODUCTION	GROWTH IN SYNTHETIC BROTH	DISTRIBUTION ON VARIOUS DIETS
Gram-positive rod	single or short chains	positive	facultative	moderate 16 hrs.	5.5-6.0	negative	poor	all diets
Curved rod	single or pairs	negative	anaerobic	slight 3-4 days	6.3-6.4	negative	poor	all diets
Short, fat rods	single	negative	anaerobic	moderate 2-3 days	6.0-6.3	negative	absent	mostly black mice on diet 101
Coliforms	single	negative	facultative	heavy, 16 hrs.	5.3-5.4	heavy	poor	mostly white mice on diet 101
Tiny coccus	long, closely united chains	positive	anaerobic	slight 2-3 days	5.9-6.0	negative	absent	mostly diet 133; few on 101
Round coccus	short chains and masses, pairs	positive	facultative	heavy, 16 hrs.	4.5-4.7	slight?	good	dextrose-containing diets
Elongate coccus	pairs and long chains	positive	facultative	heavy, 16 hrs.	4.9-5.3	negative	good	dextrin-containing diets

non ('33). The gram-positive organisms usually exhibited that reaction only in young cultures, and the cocci frequently appeared gram-negative in the original stain from the intestinal contents. The overgrowth of the slower growing anaerobes by the faster growing bacteria explains the occurrence of these anaerobes in the top dilution only, where they are found after the competing organisms have been diluted out.

These 7 types of bacteria were tested for their ability to survive 3 transfers in the absence of certain of the B vitamins omitted singly from an otherwise supposedly complete broth, and if they survived, to contribute to this broth measurable amounts of the vitamin omitted, as shown in table 2.

It will be observed from this table that 2 of the types of organisms never grew in the synthetic broth, and therefore their synthetic activities could not be tested. Two more of the bacterial types grew poorly when they survived at all. The curved rod was subcultured only twice, but both times it survived the 3 transfers in the pantothenic acid deficient broth, and was shown to liberate this vitamin into the broth, in 1 case in large quantities. The gram-positive rods that grew readily in the organic medium proved difficult to culture in the synthetic broth, and although one-third of the trials to obtain 3 transfers in synthetic broth were successful, the growth was weak. In a few cases small amounts of one of the B vitamins were found to be present in the broth. The poor growth might account for the slight synthetic activity observed.

Oddly enough the coliforms grew poorly in this synthetic broth. Known strains of *E. coli*<sup>2</sup> as well as the recently isolated cultures, gave correspondingly poor results. When the simpler, mineral salt broth was employed, growth was better, and the supernatant broth of these cultures was found to contain considerable quantities of all 5 vitamins.

A striking difference appeared when we compared the activity of the 2 facultative cocci. They both grew well in the synthetic broths, both complete and deficient, with the ex-

<sup>2</sup> Obtained through the kindness of Dr. P. B. Cowles, Department of Bacteriology, Yale University.



ception of the medium without folic acid. In this broth the coccus from animals on diets containing dextrin grew as well as on the other synthetic broths, while only 9 out of 28 cultures of the coccus from the mice fed dextrose-containing diets survived the 3 transfers in this medium, and 6 of these showed only weak growth. This difference is further emphasized by noting the data on the presence of folic acid in the supernatant broth after the cocci had been planted in the folic acid deficient broth. Of the 7 cultures tested from animals fed diets containing dextrin, 6 showed the presence of large amounts of folic acid in the supernatant broth. In contrast, the cocci from mice fed dextrose-containing diets, showed liberation of folic acid in only 4 cultures, and of these, only 1 showed the presence of large amounts.

#### DISCUSSION

The results of this preliminary study tend to indicate that differences in diet influenced the bacterial synthesis of B vitamins by the alteration of the organisms present in the intestinal tract. However, this is far from the final answer, as will be pointed out in the following discussion.

There are 2 major weaknesses in this work. The first, which is largely unavoidable with our present culturing techniques, is the valid criticism that the *in vitro* findings do not necessarily reflect the *in vivo* activities of the organism. The second weakness lies in the inability to isolate some of the predominating organisms at all, or to subculture a bacterium in synthetic broth even after primary isolation, thereby making the study of their synthetic activities impossible. Obviously further work is needed on this aspect of the problem.

This indicates that in this study, the techniques employed failed to meet the cultural requirements of all of the predominating flora. This proved to be especially true of the cecal bacteria. The conditions were more nearly met with the rich organic broth than with the synthetic medium, however, as was shown by the inability to subculture some of the primary isolations in synthetic broth, although they could be success-

fully transplanted into organic media. This suggests that for these bacteria, the synthetic broth may have had a deficiency or an imbalance.

However, the poor growth or lack of growth of some organisms in the rich organic medium indicates that there are still other flaws in the present technique. This may well be remedied by improving the means of maintaining anaerobiosis, as many of the cecal organisms were shown to be obligate anaerobes. Further work should be done in the field of culturing these organisms.

Several considerations, to be discussed here, entered into the development of the technique as it was used in these studies. In the preceding paper we have discussed our reasons for choosing the lower small intestine and the cecum for our study of their flora, since it would seem that in these areas any vitamins liberated from the cells would have the best chance of being absorbed by their host. It was also because of considerations of availability to the host that we selected the supernatant fluid of the cultures to test for the presence of the vitamins. Any vitamins held in the cells would be available to the host only if he practiced coprophagy, or if active autolysis took place in the intestinal tract at or above a point where absorption could take place. As the degree of cellular breakdown which takes place in such a region is uncertain, we tried to minimize autolysis as much as possible by using young cultures (24-hour) for testing. In most cases our cultures were increasing in turbidity between the eighteenth and twenty-fourth hour, suggesting an actively growing culture rather than a static one in which autolysis would be prevalent.

In these studies we have considered only the predominating flora of the cecal and small intestinal contents, as we felt that the bacteria present in the largest numbers would have the greatest chance to make a significant contribution to their host by any liberation of vitamins.

As has been pointed out in the preceding paper, we have not regularly found coliforms to be present as a predominating

organism except in a few white mice fed diet 101. In most cases these organisms appeared only at a much lower level of dilution. It does not seem likely that the coliforms, in spite of their good synthetic activities with respect to the 5 B vitamins tested (Thompson, '42; Burkholder and McVeigh, '42; also this paper), could contribute much to the nutrition of the mice maintained under the conditions of this study, with the exception of the few mice fed diet 101, mentioned above.

The relationship of the presence of coliforms to diet is only 1 instance of the role that diet plays in influencing the type of intestinal flora of the mouse. The difference in the type of cocci isolated from mice fed dextrose- or dextrin-containing diets is further evidence of the effect of the carbohydrate in the ration on the intestinal bacteria isolated. The importance of this difference in cocci is brought out further by the striking difference in their synthetic activities with respect to folic acid. From these results, we can assume that the coccus isolated from animals on a dextrin-containing diet was probably supplying folic acid to its host. In contrast, the coccus isolated from mice maintained on a diet containing dextrose was not elaborating any significant amount of folic acid, but apparently actually required it for growth. It was therefore probably competing with its host for any folic acid present in the diet or liberated from any other intestinal organism. Such competition for this essential nutrient might even prove detrimental to the host.

Riboflavin was found to be present in small amounts in the supernatant fluid of about one-fifth of the cultures tested. Since it has been shown (Mitchell and Isbell, '42) that riboflavin is not readily liberated from the cell, this is in keeping with the work of other investigators in this field. Although the amount liberated by each organism was small, it may be enough to become of importance when an animal is on a marginal riboflavin intake. Also any factor which would increase the number of these organisms, such as an increase in bacterial population in the cecum, should correspondingly increase the total amount of riboflavin produced. The difference in the ribo-

flavin requirement between the C<sub>37</sub> and A strain mice (Fenton and Cowgill, '47a) might be explained by the difference in bacterial population in the cecum (Gall et al., '47). A similar explanation might apply to the differences in requirements of these 2 strains for pantothenic acid (Fenton and Cowgill, '47b), since the curved rod which is a predominating organism on all 3 diets, liberated pantothenic acid both times it was successfully cultured in synthetic broth.

On the basis of these preliminary studies, it might be said that diet may influence the nutrition of the host indirectly by affecting the intestinal flora, as well as by supplying the nutrients directly.

#### SUMMARY AND CONCLUSIONS

1. A study was undertaken to determine the ability of 107 cultures, isolated from high dilutions of intestinal contents of mice fed 7 different diets, to synthesize and liberate from their cells 5 vitamins — riboflavin, niacin, biotin, folic acid and pantothenic acid, and to determine whether the synthetic products of the flora were influenced by diet.

2. A partial description was given of 7 different types of bacteria found in the top dilutions of intestinal contents of mice, with a notation as to their distribution on the various diets.

3. Differences were observed in the ability of these cultures to grow in synthetic broths, both complete and deficient in 1 of the 5 vitamins. In some instances these differences were correlated with diet, as in the case of 2 cocci. The coccus characteristic of the flora found in mice fed dextrin-containing diets, grew well in the synthetic broth lacking folic acid, in contrast to the coccus found in animals fed a diet containing dextrose, where little or no growth was observed in the folic acid-deficient broth.

4. These 2 cocci also exhibited differences in the extent of folic acid liberation; the coccus which grew well on the broth lacking folic acid liberated large amounts of this vitamin into the environment, while the coccus which grew poorly, or not



at all, in the folic acid-deficient broth, liberated little if any of this vitamin.

#### ACKNOWLEDGMENTS

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# EFFECTS OF B VITAMINS, LIVER AND YEAST ON GROWTH UNDER COLD ROOM AND ROOM TEMPERATURE CONDITIONS<sup>1</sup>

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Available data indicate that liver contains at least 1 factor other than the known B vitamins essential for optimal growth in the immature rat (Bosshardt, Ayres, Ydes and Barnes, '46; Sporn, Ruegamer and Elvehjem, '47; Jaffé and Elvehjem, '47; Cary, Hartman, Dryden and Likely, '46). Weanling rats fed purified rations containing liver invariably gained more weight than animals fed similar diets containing the B vitamins in synthetic form. These differences, although consistent for all experiments cited above, were not sufficiently marked in most cases to be statistically significant (Sporn, Ruegamer and Elvehjem, '47; Jaffé and Elvehjem, '47), particularly with diets containing unextracted casein (Cary, Hartman, Dryden and Likely, '46). The present experiment was undertaken to determine whether the above differences in growth might not be accentuated by raising animals under conditions of cold. Available data indicate that body requirements for essential nutrients may be increased by physical exertion,

<sup>1</sup> The research which this paper reports was undertaken in cooperation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the War Department.

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fever, thyroid-feeding and other conditions resulting in an increased metabolic rate (Ershoff, '48). Such "stress factors" raise body requirements beyond the usual or average range, accentuate deficiencies and hasten the onset of symptoms on a deficient diet. It was felt that since excessive doses of thyroid caused retarded growth preventable by liver feeding in the rat (Ershoff, '47a, '47b), exposure to cold with its attendant rise in oxygen consumption might also serve as a "stress factor" resulting in increased requirements for unknown growth factors in the rat.

#### PROCEDURE AND RESULTS

Four basal rations were employed in the present experiment: diets A, B, C and D. Diets A and B were purified rations containing the B complex factors in synthetic form only. Diets C and D were similar in composition but contained yeast or desiccated whole liver in addition to the synthetic vitamins. Ninety female rats of the Long-Evans strain were selected at 23 to 25 days of age at an average weight of 55 gm. Animals were kept in metal cages with raised screen bottoms to prevent access to feces and were fed *ad libitum* the diets listed in table 1. Feeding was continued for 8 weeks. Experiments were conducted (1) with animals kept continuously in a large walk-in refrigerator at a temperature of  $2 \pm 1.5^{\circ}\text{C}$ .<sup>3</sup> and (2) under standard laboratory conditions at an average temperature of approximately  $23 \pm 2^{\circ}\text{C}$ . Food consumption was determined daily for the first 4 weeks of feeding. Animals were autopsied after 8 weeks of feeding, organ weights determined, the ovaries and thyroids fixed in 10% formol, and sections prepared and stained with hematoxylin and eosin. A second experiment was conducted under conditions similar to the above except for a cold room temperature of  $6 \pm 2^{\circ}\text{C}$ . in contrast to the  $2 \pm 1.5^{\circ}\text{C}$ . employed above. Forty-eight

<sup>3</sup> Animals were kept continuously in the dark except for such times as they were being fed or weighed. Previous work indicates that exclusion of light is without significant effect on resulting pathology under cold room conditions (Kenyon, '33).

female rats of the Long-Evans strain were weaned at 21 to 23 days of age and fed diets A, C and D, both under cold room and room temperature conditions. Feeding was continued for 8 weeks. During the eighth week of feeding metabolic rates were determined for all rats in both the cold room and room temperature series.

TABLE 1  
*Composition of experimental diets.*

DIETARY COMPONENT	DIET A	DIET B	DIET C	DIET D
Yeast <sup>1</sup>	0.0	0.0	10.0	0.0
Whole liver powder <sup>2</sup>	0.0	0.0	0.0	10.0
Vitamin test casein <sup>3</sup>	22.0	22.0	22.0	22.0
Salt mixture <sup>4</sup>	4.5	4.5	4.5	4.5
Sucrose	73.5	73.5	63.5	63.5

To each kg of diet A, C and D were added the following synthetic vitamins: thiamine hydrochloride 72 mg, riboflavin 9 mg, pyridoxine hydrochloride 15 mg, calcium pantothenate 67.2 mg, nicotinic acid 60 mg, 2-methyl-naphthoquinone 5 mg and choline chloride 1.2 gm.

To each kg of diet B were added thiamine hydrochloride 144 mg, riboflavin 18 mg, pyridoxine hydrochloride 30 mg, calcium pantothenate 134.4 mg, nicotinic acid 120 mg, inositol 1.2 gm, p-aminobenzoic acid 600 mg, folic acid 10 mg, biotin 1 mg, 2-methyl-naphthoquinone 10 mg and choline chloride 1.2 gm.

Each rat also received 3 times weekly the following supplement: cottonseed oil (Wesson) 500 mg, alpha-tocopherol 1 mg, and a vitamin A-D concentrate <sup>5</sup> containing 50 U.S.P. units of vitamin A and 5 U.S.P. units of vitamin D.

<sup>1</sup> Brewers' Type Yeast no. 200, Anheuser-Busch, Inc., St. Louis, Mo.

<sup>2</sup> Whole Dried Liver Powder, Armour and Co., Chicago, Ill.

<sup>3</sup> Vitamin Test Casein, General Biochemicals, Inc., Chagriu Falls, Ohio.

<sup>4</sup> Salt Mixture no. 1 (Sure, '41).

<sup>5</sup> Nopco Fish Oil Concentrate, assaying 800,000 U.S.P. units of vitamin A and 80,000 U.S.P. units of vitamin D per gram.

Findings are summarized in table 2. Data for the cold room series were computed on the basis of the top 8 animals in each group (originally 12 rats per group) in the first experiment and for the top 7 animals per group (initially 10 rats per group) in the second experiment to minimize variations in averages due to early deaths, infection and atypical responses on the part of individual rats. Growth was significantly reduced in all rats under cold room conditions; animals fed

liver (diet D), however, gained significantly more weight than those fed synthetic rations (diets A and B) with growth on the yeast-containing ration (diet C) intermediate between the two.<sup>4</sup> At room temperature findings confirm those of earlier workers (Sporn, Ruegamer and Elvehjem, '47; Jaffé and Elvehjem, '47). Animals fed liver gained more weight than

TABLE 2

*Effects of B vitamins, liver and yeast on growth of rats maintained under cold room and room temperature conditions.*

GROUP DIETARY	FIRST EXPERIMENT			SECOND EXPERIMENT		
	Number of animals	Average initial body weight	Average gain in body weight over 8-wk. period <sup>1</sup>	Number of animals	Average initial body weight	Average gain in body weight over 8-wk. period <sup>1</sup>
Cold room series						
		<i>gm</i>	<i>gm</i>		<i>gm</i>	<i>gm</i>
A	8	54.4	88.4 ± 2.8	7	50.1	84.9 ± 3.1
B	8	56.0	93.0 ± 3.9			
C	8	55.6	99.3 ± 3.8	7	49.8	91.6 ± 3.7
D	8	56.0	111.5 ± 5.0	7	50.3	112.7 ± 4.8
Room temperature series						
A	6	53.8	146.0 ± 7.3	6	49.6	142.3 ± 8.1
B	6	53.9	155.4 ± 9.6			
C	6	53.7	159.0 ± 8.1	6	49.4	152.8 ± 8.5
D	6	53.7	167.7 ± 9.9	6	49.8	160.5 ± 10.2

<sup>1</sup>Including standard error of the mean calculated as follows:  $\sqrt{\sum d^2/n}/\sqrt{n}$  where "d" is the deviation from the mean and "n" is the number of observations.

those fed synthetic or yeast-containing rations. At room temperature, however, these differences were in no case sufficiently pronounced to be statistically significant.

Data on food consumption and the relative efficiency of the various diets for the building of body tissue are summarized in table 3. The findings indicate that for the first 4 weeks of feeding relative efficiency as measured by the ratio of the gain

<sup>4</sup>The highest values obtained on diet A were less than the average on diet D. In both experiments the quotient of M.D./S.E.M.D. for diets A and D was in excess of 4. Values of 3 or larger are considered statistically significant.

TABLE 3  
Summary table showing average gain in weight, average food consumption and ratio of increase in weight to calories consumed for the first 28 days of feeding.

DIETARY GROUP	INITIAL BODY WEIGHT	AVERAGE GAIN IN BODY WT. <sup>1</sup>	FOOD CONSUMPTION (GM/DAY) ON FOLLOWING WEEKS OF EXPERIMENT <sup>2</sup>				AVERAGE TOTAL FOOD INTAKE PER RAT FOR FIRST 4 WEEKS OF FEEDING <sup>1,2,3</sup>	EFFICIENCY <sup>4</sup>
			1st	2nd	3rd	4th		
Cold room series (8 animals per group)								
		gm					gm	calories
A	54.4	51.8 ± 2.3	12.7	14.8	15.5	16.9	419.6 ± 8.5	1552.5 ± 31.5
B	56.0	54.0 ± 2.3	13.2	16.9	17.1	18.0	457.0 ± 9.2	1690.9 ± 34.0
C	55.6	53.8 ± 3.4	12.8	15.5	16.2	17.9	435.2 ± 11.1	1610.2 ± 41.1
D	56.0	63.7 ± 3.3	13.0	16.4	17.1	18.0	451.1 ± 12.3	1669.0 ± 45.5
Room temperature series (6 animals per group)								
A	53.8	91.3 ± 4.6	8.9	10.5	11.6	11.7	298.8 ± 3.7	1105.6 ± 13.7
B	53.9	104.7 ± 5.2	9.5	11.1	12.6	13.2	324.6 ± 12.8	1201.0 ± 47.5
C	53.7	111.5 ± 5.0	9.6	11.6	12.5	13.0	325.8 ± 13.6	1205.5 ± 50.4
D	53.7	120.2 ± 8.6	9.8	12.2	13.2	13.7	341.8 ± 16.1	1264.7 ± 59.2
								8.19 ± 0.41
								8.70 ± 0.20
								9.22 ± 0.28
								9.45 ± 0.30

<sup>1</sup> Including standard error of the mean calculated as follows:  $\sqrt{\sum d^2/n}/\sqrt{n}$  where "d" is the deviation from the mean and "n" is the number of observations.

<sup>2</sup> These values are based on basal ration only and do not include the cottonseed oil ingested in supplementary feedings.

<sup>3</sup> The calorie value of diets A, B, C or D approximated 3.7 calories per gram of basal ration.

<sup>4</sup> Gm increase in weight  $\times 100$ .  
Calories consumed

in weight  $\times 100$  to the calories consumed was slightly higher for animals fed liver (diet D) both under cold room and room temperature conditions than for those fed other diets tested. In most cases, however, these differences were not statistically significant. Values for the cold room series were less than for animals maintained at room temperature conditions. These differences were due, at least in part, to increased heat loss under cold room conditions.

TABLE 4

*Body and organ weights of rats maintained under cold room and room temperature conditions.*

DIETARY GROUP	INITIAL BODY WEIGHT	BODY WEIGHT AFTER 8TH WEEK OF FEEDING	VENTRICULAR WEIGHT	KIDNEY WEIGHT	OVARIAN WEIGHT	ADRENAL WEIGHT
Cold room series (8 animals per group)						
	<i>gm</i>	<i>gm</i>	<i>mg</i>	<i>gm</i>	<i>mg</i>	<i>mg</i>
A	54.4	142.8	785	1.678	35.4	40.0
B	56.0	149.0	733	1.634	37.0	36.6
C	55.6	154.9	770	1.832	32.5	39.2
D	56.0	167.5	826	1.868	40.3	51.1
Room temperature series (6 animals per group)						
A	53.8	199.8	640	1.602	46.1	46.0
B	53.9	209.3	659	1.664	52.7	44.2
C	53.7	212.7	637	1.673	55.5	49.7
D	53.7	221.4	649	1.758	54.3	59.5

At autopsy significant differences were observed between animals raised under cold room and room temperature conditions (table 4). Not only was growth retarded in the former series but approximately half the rats in this group lost part or all of their tails during the 8-week feeding period;<sup>5</sup> ventricular weight was increased not only relative to body weight but in absolute weight as well; kidney weights were increased relative to body weight; thyroids were similar to those described by Kenyon ('33) with cellular hypertrophy and loss of colloid; and approximately one-third of the rats

<sup>5</sup> Apparently due to poor circulation in the tail with resulting necrosis and sloughing off of the affected tissue.

had ovaries infantile both in weight and microscopic appearance. With the exception of growth, animals raised under cold room conditions did not differ significantly from one another on any of the diets employed.

Data on oxygen consumption for animals raised under cold room and room temperature conditions are summarized in table 5.<sup>6</sup> The apparatus employed in making these determinations was a closed circuit type with a capacity of 2 liters

TABLE 5  
*Oxygen consumption of rats maintained under cold room  
and room temperature conditions.*

DIETARY GROUP	NUMBER OF ANIMALS	O <sub>2</sub> CONSUMPTION ML/HR/100 GM BODY WEIGHT <sup>1</sup>	INCREASE OVER B.M.R. <sup>2</sup>
Cold room series			
			%
A	7	252 ± 17	92.4
C	7	205 ± 12	50.7
D	7	198 ± 8	55.9
Room temperature series			
A	6	131 ± 5	...
C	6	136 ± 5	...
D	6	127 ± 4	...

<sup>1</sup> Including standard error of the mean calculated as follows:  $\sqrt{\sum d^2/n}/\sqrt{n}$  where "d" is the deviation from the mean and "n" is the number of observations.

Values for O<sub>2</sub> consumption in ml/hr/100 gm body weight are somewhat greater for the room temperature series than those usually reported as basal for the rat. These differences may be due in part to a strain difference.

<sup>2</sup> Compared to the control B.M.R. in the room temperature series.

(Mason and Winzler, '47). Carbon dioxide was absorbed with sodium hydroxide, and oxygen consumption was determined from pressure changes recorded by means of a water manometer. Readings were made during the eighth week of feeding, and for the cold room series at a temperature of 6°C. with apparatus present in the same room in which animals were housed. In the room temperature series respiration chambers

<sup>6</sup> We are indebted to Mr. G. D. Mason for these determinations.



were kept at 28°C., and in both cases readings obtained were corrected to standard temperature and pressure. At least 6 successive 5-minute intervals were recorded for each animal, with care being taken to record oxygen consumption when animal activity was at a minimum. Findings in table 5 indicate that average oxygen consumption in ml/hr/100 gm body weight was at least 50% greater for the cold room rats than were values obtained at 28°C. for the room temperature series.<sup>7</sup> In the cold room series values for diet A were greater than for rations containing liver or yeast; there is a question, however, whether these differences are significant. In the room temperature series no significant difference in metabolic rate was noted on the various diets employed.

#### DISCUSSION

Since the differences in growth between the synthetic and liver-containing diets were significant under cold room but not room temperature conditions, it is felt that the increased metabolism resulting from exposure to low environmental temperature increased requirements for 1 or more nutrients present in liver but not present in significant amounts in other diets employed. The increased requirements under cold room conditions accentuated tissue deficiencies on the synthetic diets employed and resulted in a gain in body weight significantly less than that observed when liver was added. Findings in the present experiment indicate that the protective factor was distinct from any of the known B vitamins and that it was not present in significant amounts in yeast. Liver, however, counteracted only part of the growth retardation observed under cold room conditions. This finding indicates either that (1) insufficient amounts of liver were employed, (2) the retardation of growth was due to factors other than of nutri-

<sup>7</sup> The increase in metabolism due to exposure to low environmental temperature was less in the present series than that reported by other investigators (Benedict and MacLeod, '29). This difference may be due in part to adaptation during our prolonged feeding period as well as to possible differences in the strain of rat employed.

tional origin, or (3) that the animals raised under cold room conditions were deficient in nutrients other than those listed above.

Available data indicate that rats raised under cold room conditions differed in several important respects from those fed excessive doses of thyroid although in both cases oxygen consumption was significantly increased. Both growth retardation and total caloric intake were significantly greater for animals maintained at cold room conditions than for those fed massive doses of thyroid, although oxygen consumption in ml/hr/100 gm body weight was greater for the latter (Ershoff, '47b). In both groups ventricular weight was increased, but no reduction was observed in myocardial creatine under cold room conditions (Brunish and Ershoff, '47) such as occurred in animals fed thyroid (Bodansky, '35; Ershoff, '47b). Finally, liver completely counteracted the retardation of growth of animals fed massive doses of thyroid (Ershoff, '47a, '47b), while such was not the case with the retarded growth occurring under cold room conditions.

#### SUMMARY

Immature female rats were raised to maturity under cold room and room temperature conditions on purified rations containing the B vitamins as synthetic factors and as present in whole liver and yeast. Growth was markedly reduced in all rats under cold room conditions; animals fed liver, however, gained significantly more weight than those fed other diets employed. The protective factor(s) was distinct from any of the known B vitamins and was not present in significant amounts in yeast. At room temperature conditions no significant difference in growth occurred on any of the diets tested. The suggestion is made that whole liver contains at least 1 factor other than the known B vitamins whose requirement is increased in animals maintained under cold room conditions.

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# EFFECT OF VEGETARIAN SELF-SELECTION DIETS ON REPRODUCTION AND THE GROWTH OF OFFSPRING OF RATS <sup>1</sup>

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In a preceding paper ('47), we described vegetarian self-selection diets (diets 6, 6C and 6CC) which were used in studies of the growth and longevity of rats. These diets differed only in regard to the fresh leafy vegetable supplements that were used. Diet 6 was supplemented mainly by lettuce, diet 6C by lettuce and celery cabbage, and diet 6CC by celery cabbage only. The present paper concerns the effects of these diets on the reproduction and growth of offspring of rats.

## METHOD OF STUDY

First, the effect of diet 6 on the reproduction and growth of offspring of 3 generations of Wistar rats was determined. After that, the effect of diets 6C and 6CC on 3 generations of Wistar and Sprague-Dawley rats was determined. In these tests, the rats were inbred by brother-sister matings so far as possible. In general 2 females were bred with each male. The aim was to obtain 3 litters from each fertile female on the vegetarian diets and to use only members of second or third litters for continuing the lines. However, in some cases members of first litters were used for further breeding because no members of later litters were raised. Moreover,  $F_2$  generation females were mated with  $F_1$  males when  $F_2$  males of the

<sup>1</sup> This study was aided by a grant from Swift & Co., Chicago.

same strain seemed to be sterile, and finally  $F_2$  Wistar females were mated with Sprague-Dawley males because all of the Wistar males that were reserved for breeding died. Litters of more than 8 young were reduced to 8 a few days after the litters were cast. After 3 litters were obtained on the vegetarian diet or the rats appeared to be sterile, they were fed an omnivorous diet (generally our basal omnivorous diet 1 [47]), and its effect on the reproduction and growth of offspring was determined.

## RESULTS

Data concerning the reproductive performance of the rats and the weights of their offspring at 42 days are presented in table 1. For comparison purposes, data are included in the table for rats fed the vegetarian diets, and for breeding rats fed our omnivorous diets. Data obtained on the 2 strains of

TABLE 1

*Reproductive performance of rats on omnivorous and vegetarian diets and weights of offspring at 42 days.*

GENERATION <sup>1</sup>	MALES MATED		FEMALES MATED		LITTERS CAST	INDIVIDUALS RAISED	MALES RAISED		FEMALES RAISED	
	Fertile		Fertile				Av. wt. at 42 days		Av. wt. at 42 days	
	no.	%	no.	%			no.	gm	no.	gm
Rats on omnivorous diet										
F <sub>0</sub> to F <sub>3</sub>	14	64	28	68	38	61	92	161	112	134
Rats on vegetarian diets										
F <sub>0</sub>	9	89	18	94	49	21	39	66	43	62
F <sub>1</sub>	8	75	14	100	37	13	19	50	17	47
F <sub>1</sub> ♂ × F <sub>2</sub> ♀	2	100 <sup>2</sup>	4	100	4	19	3	44	2	39
F <sub>2</sub>	4	50	6	50	3	0				
Rats on omnivorous diet after vegetarian diet										
F <sub>0</sub> to F <sub>2</sub> <sup>3</sup>	12	83	19	95	22	71	63	155	60	130

<sup>1</sup>  $F_0$  generation omnivores means rats previously on a stock diet.  $F_0$  generation vegetarians means offspring of rats fed omnivorous diets.

<sup>2</sup> Previously known to be fertile.

<sup>3</sup> Generations of vegetarians.

rats with the differently supplemented vegetarian diets are combined as no significant differences in the results were noted. As indicated, most of the rats on the vegetarian diets were fertile but they raised less than half the percentage of offspring raised by the rats fed our omnivorous diets, and these offspring of the vegetarians weighed less than half as much as those of the omnivores. Moreover, these young of the vegetarians weighed less in successive generations while those of the omnivores tended to be of equal weight. After the

TABLE 2

*Season of the year in relation to type of diet, number of offspring born, and per cent of young raised.*

DIET AND OFFSPRING BORN	SEASON OF YEAR			
	Dec.—Feb.	Mar.—April	May—Aug.	Sept.—Nov.
<i>Omnivorous:</i>				
Number born <sup>1</sup>	69	71	86	116
% raised	41	52	83	59
<i>Vegetarian:</i>				
Number born <sup>1</sup>	187	171	178	197
% raised	0	17	37	17
<i>Omnivorous <sup>2</sup>:</i>				
Number born <sup>1</sup>	47	57	46	22
% raised	74	56	85	73

<sup>1</sup> These are the number of rats born less the number sacrificed to reduce the size of litters of more than 8 new-born.

<sup>2</sup> After vegetarian diet.

vegetarians were fed one of our omnivorous diets, they raised a higher percentage of young than did the rats that were kept on our omnivorous diets, and their offspring immediately approached the weights of those of the omnivores. In fact, the male rat that became heaviest at 42 days (208 gm) was an offspring of ex-vegetarians.

Table 2 shows that the rats on the vegetarian diets raised no offspring in winter (3 winters included) and only 37% in summer. The omnivorous controls revealed a parallel but less extreme seasonal variation in the percentage of offspring

raised, but with respect to the vegetarians that were finally fed an omnivorous diet no significant seasonal influence on the percentage of offspring raised was observed.

Some of the offspring of the vegetarians were deformed at an early age but the deformities were evidently mainly due to the development of severe rickets. The inclusion of celery cabbage as a source of calcium (as well as of supplementary protein in the diet) did not prevent the development of severe rickets in some cases. At autopsy, enlarged joints and bowed fibula or tibia were found and the femurs of over half of the offspring of the vegetarians could not be removed without a separation of the head of the femur, due to poor calcification. This occurred even in offspring of the vegetarians that were over 200 days old and that had been fed our basal omnivorous diet (which included 3% bone meal) 30 days or more. Many of the offspring of the vegetarians were also more hyperexcitable and manifested more fear of being handled than did the offspring of rats on omnivorous diets, and the hypersensitivity did not decrease after the vegetarians were placed on an omnivorous diet. The sexual development of the vegetarians was retarded, and more so in the males than in the females. The feces of the vegetarians were often poorly formed.

#### DISCUSSION

The low percentage of offspring raised by the rats on the vegetarian diets was due to a high neonatal mortality and the apparent killing and eating of a large percentage of the young by the mothers. The killing of a pup by a vegetarian mother (by biting into the nape of the neck) was directly observed only once, but as many as 7 otherwise seemingly viable young were eaten by the vegetarian mothers during a single night. Occasionally, rats fed diets including meat also kill and eat their offspring (as observed) but the eating of a much larger percentage of offspring by our vegetarians was most likely due to the deficiencies in the vegetarian diets of suitable protein, calcium and other nutritional essentials. It is conceivable that the normal eating of the placentas after the

young are born whets the appetite of rats in need of such food and leads them to eat more than merely the placentas.

The failure of the vegetarians receiving celery cabbage to raise more offspring or heavier offspring than did the vegetarians receiving mainly lettuce as a fresh leafy vegetable supplement to the diet may be explainable by the relatively small amount of celery cabbage eaten. The rats ate much more lettuce than celery cabbage when both were supplied and they ate only slightly more celery cabbage when no lettuce was supplied. Some rats ate practically no celery cabbage, and the third generation of rats that were fed celery cabbage as the sole fresh leafy vegetable supplement to the diet still preferred lettuce when this was finally supplied. The lettuce fed by us consisted mainly of trimmings of head-lettuce (the green outer leaves and cuttings of the stems). The rats preferred this to leaf-lettuce, spinach, cabbage, celery, broccoli, cauliflower trimmings, carrot tops and beet tops. Variable amounts of greens other than lettuce were nevertheless usually also eaten when supplied with lettuce.

Considerable individual differences in the preferences or acceptances of the different items included in the basal part of the vegetarian self-selection diets were manifested by the rats, and changes in preferences occurred with ageing, pregnancy and in successive generations. The general order of preferences or acceptances of the different items was, however, approximately as follows: the germ part of kernels of corn, sunflower seeds, peanuts, green peas, the end of kernels of corn opposite the germ, defatted corn germ meal, barley, wheat, rolled oats, alfalfa leaf meal, soy beans, brewers' yeast, defatted wheat germ meal, salt (NaCl). The relatively low acceptance value of soy beans may help to explain why soy beans have not been used to any great extent even in China (according to Adolph, '44). Of special interest seemed to be the gnawing of the end of kernels of corn opposite the germ, mainly by young rats in the  $F_1$  and  $F_2$  generations. This end of the kernels of corn forms the surface of the ears and, as a result of pervaporation in the drying of the ears, the



surface evidently contains a concentration of water-soluble and presumably tasty components (Alexander, '33). The preference of the apparently more tasty part of the kernels of corn to the germ part suggests a finicky appetite. However, a sharp change to a preference of the germ part occurred in the females when they became pregnant. The general preference for the germ part of corn, sunflower seeds and peanuts to other items on the diet incidentally suggests that the fat or oil in these foods partly explains their appeal to the rats.

The period between weaning (at 35 days) and sexual maturity (as late as 150 days in some vegetarians) appeared to be the most difficult for the offspring of the vegetarians to bridge. About half of the males died during this period. The females fared better. Under more natural environmental conditions than those under which rats are kept in the laboratory they would evidently thrive better on an otherwise purely vegetarian diet as they would be able to resort to geophagy, osteophagy, the eating of insects and the eating of all dead rats. We removed dead rats, partly eaten rats and dying rats to prevent their being eaten. There was no evidence, however, of any tendency of adult vegetarians to resort to coprophagy as shown by rats on some vitamin deficient diets.

In contrast to the results of our study, Wu and Chen ('29) using a simple vegetarian diet supplemented alternately by colza and (Chinese) "small cabbage," found that their rats raised as high a percentage of offspring in the first 3 generations as rats fed their omnivorous stock diet. Their strain of rats may have been better adapted to living on a purely vegetarian diet than the strains we used, but it seems more likely that prolonged human experience of living on practically vegetarian diets in China has led to the cultivation of vegetables like colza and "small cabbage" which serve better as supplements to grains and legumes than leafy vegetables commonly raised in the United States. Moreover, the soil and agricultural methods used in China may serve to produce foods (grains and legumes as well as leafy vegetables) of

higher biological value than similar foods grown in the United States. Wu and his associates eventually raised 25 (or more) generations of rats with their vegetarian diet but the growth of the rats was subnormal and, after the first generation, 70% developed cataracts (Chen, Chang and Luo, '41). A purely vegetarian diet satisfactory for albino rats therefore still remains to be found.

#### SUMMARY

Rats on vegetarian self-selection diets including corn (whole kernels), wheat (whole grains), pearled barley, rolled oats, sunflower seeds, peanuts, soy beans, green peas, corn germ meal, wheat germ meal, brewers' yeast, alfalfa leaf meal, salt (NaCl), lettuce and celery cabbage were found to be generally fertile but raised less than 25% of their offspring. They raised none in winter. The growth of these young was also subnormal and severe rickets commonly developed.

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# RELATION OF CARBOHYDRATE TO INTESTINAL SYNTHESIS OF BIOTIN AND HATCHABILITY IN MATURE FOWL<sup>1, 2</sup>

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## FIVE FIGURES

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It is well-recognized that changes in the kind of dietary carbohydrate may be reflected in the amount of vitamins synthesized by intestinal microorganisms (Guerrant, Dutcher and Tomey, '35; Morgan, Cook and Davison, '38; Mannering, Orsini and Elvehjem, '44; Najjar and Barret, '45; and Teply, Krehl and Elvehjem, '47). Evidence has been presented which shows that biotin is synthesized in animals (McElroy and Jukes, '40; Wegner et al., '41; Nielsen et al., '42; Mitchell and Isbell, '42) and in humans (Gardner et al., '43, '45, '46; Oppel, '42); it is synthesized by the bacteria of the intestinal tract (Landy et al., '42; Thompson, '42; Burkholder and McVeigh, '42).

The laying hen appeared to be a desirable experimental animal for studying intestinal synthesis since the vitamin content of the egg could be determined and would be a measure of the biotin available for metabolism. The use of the egg

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<sup>3</sup> General Education Board Fellow.

as a measure of intestinal synthesis eliminates certain difficulties associated with collecting and assaying urine and feces in the chicken. Cravens, Sebesta, Halpin and Hart ('42) reported that biotin was necessary for normal embryonic development in the domestic fowl.

The present investigation was initiated to determine the effect of different carbohydrates on intestinal synthesis of biotin in the mature fowl. Sucrose, dextrin, and combinations of sucrose with lactose or dried whey were tested. Egg production, hatchability of fertile eggs, and biotin content of the eggs were the criteria used in evaluating the extent of intestinal synthesis and absorption of biotin.

#### MATERIALS AND METHODS

Twenty-four Single Comb White Leghorn pullets were placed in individual laying cages with raised screen bottoms, after being reared on the Experiment Station range. All birds were artificially inseminated weekly with mixed semen from New Hampshire cockerels. All eggs laid were marked with the hen's number and the date and settings were made weekly. The eggs were candled on the sixth and seventeenth days of incubation, at which times the infertile eggs and dead embryos were removed. All eggs which were removed on candling, and also those which failed to hatch, were broken and the age of the embryo at the time of death was estimated. Any gross abnormalities were recorded.

The pullets were fed an all-mash laying ration for 4 weeks prior to the start of the experiment, during which period data were collected on egg production and hatchability. At the end of this pre-experimental period the 24 birds were divided into 6 groups of 4 pullets each. At the end of the ninth week of the experimental period, the pullets which had been receiving diets B33 and B35 were placed on the practical all-mash diet and received the latter during the final week of the experiment. This change was necessitated by the poor condition of the birds at the end of the ninth week.

The materials used in this study were sucrose, dextrin (prepared by autoclaving moist cornstarch 2 hours with subsequent drying and grinding), lactose and dried whey. The composition <sup>4</sup> of the diets is shown in table 1. Crystalline B-vitamins were added in amounts which were thought to meet

TABLE 1  
*Composition of diets.*

	B31	B32	B33	B34	B35
Sucrose — %	63		48	63	38
Dextrin — %		63			
Purified casein — %	18	18	18	18	18
Gelatin — %	5	5	5	5	5
Salts IV — %	5	5	5	5	5
Liver fraction “L” — %	4	4	4	4	4
Fish oil (3000 A) (400 D) — %	2	2	2	2	2
Soybean oil — %	3	3	3	3	3
Lactose — %			15		
Dried whey — %					25
Choline — %	0.2	0.2	0.2	0.2	0.2
Oyster shell			Ad libitum		
All vitamins listed below added as indicated in mg per kg of ration					
Biotin				0.2	
Thiamine HCl	4	4	4	4	4
Riboflavin	6	6	6	6	6
Ca pantothenate	15	15	15	15	15
Niacin	100	100	100	100	100
2-methyl-1,4-naphthoquinone	0.5	0.5	0.5	0.5	0.5
Pyridoxine HCl	4	4	4	4	4
Alpha tocopherol	3	3	3	3	3

the requirements of the pullets with the exception of biotin and pteroylglutamic acid. Liver fraction "L" was used as a source of the latter vitamin. Biotin was omitted from all diets except B34 since this vitamin was the one under study. A practical all-mash diet was used as a positive control in addition to diet B34. Oyster shell and tap water were supplied ad libitum.

<sup>4</sup> We are indebted to the Western Condensing Co., for the lactose and dried whey; to Wilson Laboratories, Chicago, Illinois, for the liver fraction "L"; to Merck and Co., Rahway, N. J., for the biotin.

Eggs which were to be assayed for biotin were set aside on the last 2 days of each week. The microbiological method of Luckey, Moore and Elvehjem ('46), with slight modification, was used. The egg whites and egg yolks were separated and those from each group of birds were pooled for assay. After thorough mixing, approximately 10 gm were weighed out. An equal number of milliliters of 4N sulfuric acid was added and the sample autoclaved for 2 hours at 15 pounds pressure. In preliminary assays on eggs it was determined that hydrolysis with 4N, 8N or 12N sulfuric acid, as outlined above, gave approximately the same results. The 4N acid was selected since the sample would contain sodium sulfate on neutralization. After autoclaving, the pH was adjusted to 4-5 with 4N sodium hydroxide and the samples were filtered through Whatman no. 1 filter paper. The filtrate was neutralized to pH 6.8-7.0 and preserved under toluene in the refrigerator. Dilutions were made as needed. The organism used for the assay was *Streptococcus faecalis* R. The extent of growth of the organism was measured by turbidity using the Evelyn colorimeter. Satisfactory recoveries of biotin were obtained when known amounts of the vitamin were added to the samples. The biotin content of the egg whites and yolks is expressed in millimicrograms per gram on a fresh weight basis.

#### EXPERIMENTAL RESULTS

*Egg production.* The production of pullets fed the various diets is shown in figure 1. The egg production of pullets fed diets B31 and B32 and those fed the practical all-mash diet followed the same general trend throughout the 10-week period although a slight drop in the egg production of pullets fed sucrose, diet B31, may be noted during the last week of the experiment. The egg production of pullets fed sucrose plus biotin, diet B34, was somewhat erratic. The fluctuations in this case were traceable to 1 pullet and are more apparent due to the small number of pullets in the group; this pullet would lay a clutch of eggs and go out of production, then come back in production and repeat the same procedure.

The rather high levels of lactose and dried whey appeared to cause a decrease in egg production. The production of pullets fed diets B33, lactose, and B35, dried whey, decreased during the third and fourth weeks of the experimental period and remained at a fairly low level for the rest of the 10-week period. This low production was unfortunate because an insufficient number of eggs were laid to obtain a measure of

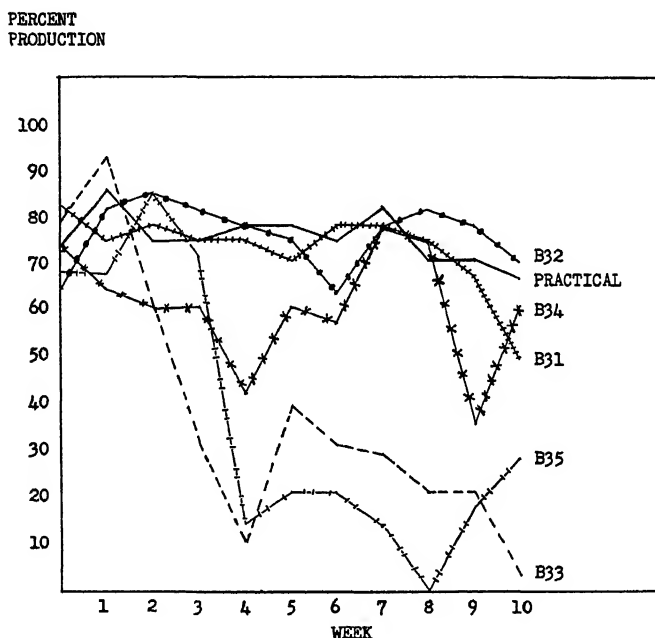


Fig. 1 Effect of diet on egg production: diet B31, sucrose; B32, dextrin; B33, lactose; B34, biotin; B35, dried whey.

hatchability and for biotin assay in the case of pullets fed dried whey (diet B35).

*Hatchability.* The per cent hatchability of fertile eggs is shown in figure 2. Hatchability of eggs from pullets fed diet B31, sucrose, decreased to zero by the third week and remained at this level. Hatchability of eggs from pullets fed diet B32, dextrin, ranged from 60 to 85% during the 10-week period. This was somewhat lower than that of the positive control



groups which were fed diet B34 and the practical all-mash diet. Hatchability of eggs from pullets fed diet B34, sucrose plus biotin, was approximately the same as that of those fed the practical all-mash diet. The results with respect to hatchability obtained with diets B33, lactose, and B35, dried whey, were somewhat inconclusive after the third and fourth weeks due to the poor egg production which resulted from the

PERCENT  
HATCHABILITY

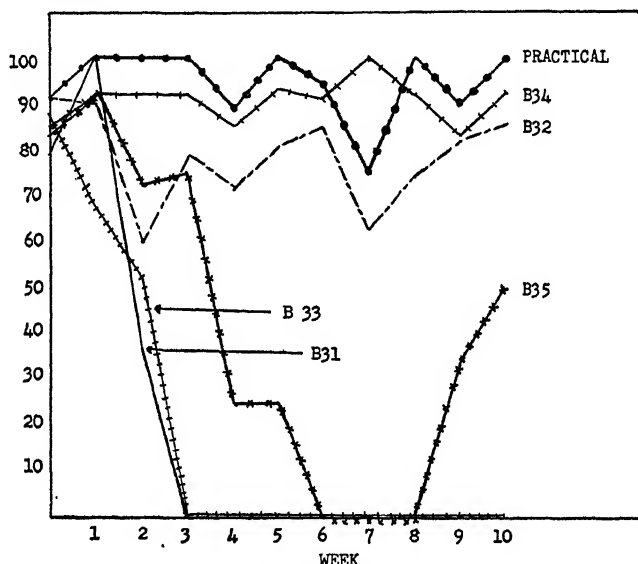


Fig.2 Effect of diets on hatchability: diet B31, sucrose; B32, dextrin; B33, lactose; B34, biotin; B35, dried whey.

amounts of these materials used in this study. This was discussed above.

*Biotin content of egg white and yolks.* At the beginning of the experiment the biotin content of the egg whites (fig. 3) varied from 65 to 105  $\mu\text{g}$  per gram expressed on a fresh weight basis. The biotin content of egg whites from hens fed diet B31 decreased from 65 to 17  $\mu\text{g}$  per gram during the first week and the presence of the vitamin was not detected in egg whites from these birds after this time. The level of biotin in

the whites of eggs from pullets fed diet B32 decreased from 88 to 22  $\mu\text{g}$  per gram during the first week and remained at the latter figure until the end of the third week; during the fourth week there was a further decrease from 22 to about 4-5  $\mu\text{g}$  of biotin per gram and it remained at this level through the eighth week.

MILLIMICROGRAMS  
BIOTIN PER GRAM

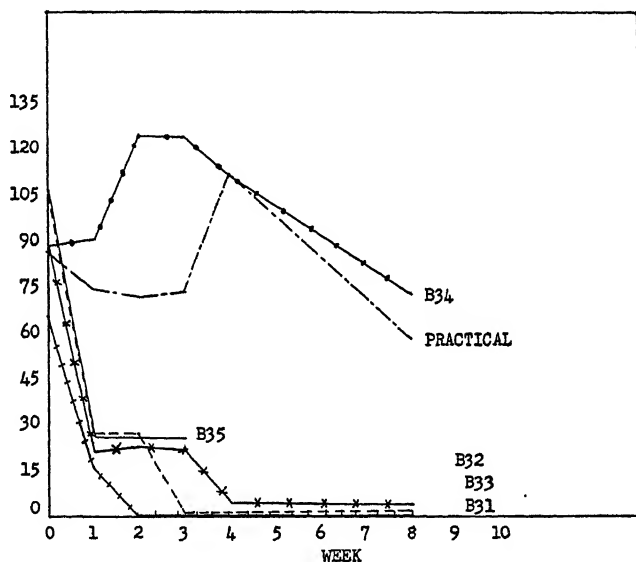


Fig. 3 Effect of diet on biotin content of egg white: diet B31, sucrose; B32, dextrin; B33, lactose; B34, biotin; B35, dried whey.

The biotin content of egg whites from pullets fed diet B33 decreased from 105 to 27  $\mu\text{g}$  per gram during the first week and remained at the latter figure throughout the second week. At the end of the third week and thereafter, biotin could not be detected in egg whites from pullets fed diet B33. There was a decrease in the biotin content of egg whites from pullets fed diet B35 from 105 to 26  $\mu\text{g}$  of biotin per gram during the first week and it remained at the latter figure until the end of the third week. No eggs were analyzed from

pullets fed diet B35 after the third week due to the poor egg production of the birds. The feeding of 200  $\mu$ g of biotin with sucrose, diet B34, was sufficient to maintain a slightly higher level of the vitamin in egg whites than was maintained in egg whites of pullets fed the practical all-mash diet (fig. 3).

The egg yolks from the pullets used in this study contained approximately 500 m $\mu$ g of biotin per gram at the start of the

MILLIMICROGRAMS  
BIOTIN PER GRAM

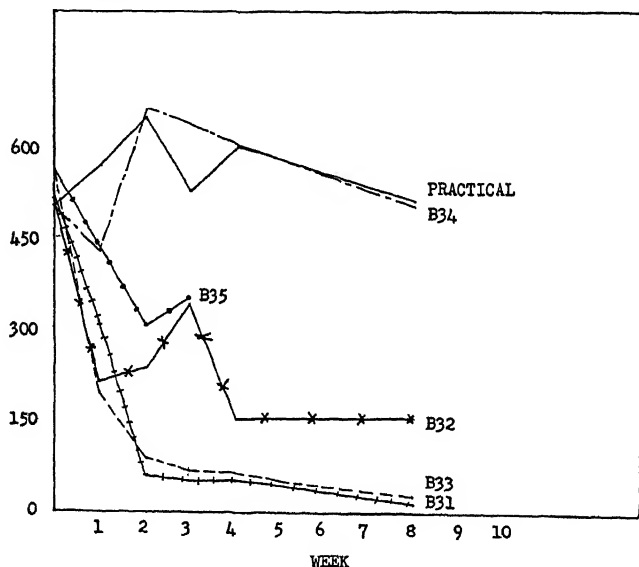


Fig. 4 Effect of diet on biotin content of egg yolk: diet B31, sucrose; B32, dextrin; B33, lactose; B34, biotin; B35, dried whey.

experiment (fig. 4). The biotin content of the yolks from pullets fed diets B31, sucrose, and B33, lactose, decreased from about 500 to 50–70 m $\mu$ g of biotin per gram during the first 2 weeks of the experiment. The egg yolks from pullets fed diet B32, dextrin, showed a decrease of from 500 to approximately 150 m $\mu$ g of the vitamin per gram and remained at the lower level. The biotin content of egg yolks from pullets fed diet B35 followed the same general trend as those

TABLE 2

*Effect of diet on incidence of syndactyly and other skeletal deformities.*

HEN	DIET	NO. OF FERTILE EGGS	EMBRYOS DYING		NUMBER OF EMBRYOS SHOWING			SYN- DACTYLY
			Before 8 days	After 8 days	Beak par- rot	Crooked tibia	Short twisted tarsus meta- tarsus	
1	B-31	49	19	18	8	7	7	7
2		13	3	7	3	6	6	6
3		16	14	0	0	0	0	0
4		35	18	14	8	2	2	5
Total		113	54	39	19	15	15	18
5	B-32	40	2	2	0	0	0	0
6		48	2	6	0	0	0	0
7		49	2	4	0	0	0	0
8		43	7	9	0	0	0	0
Total		180	13	21	0	0	0	0
9	B-33	28	9	11	3	5	5	4
10		13	7	1	0	0	0	0
11		11	1	4	4	4	4	3
12		9	1	7	1	2	2	4
Total		61	18	23	8	11	11	11
13	B-34	37	2	3	0	0	0	0
14		42	1	0	0	0	0	0
15		33	0	4	0	0	0	0
16		6	0	0	0	0	0	0
Total		118	3	7	0	0	0	0
17	B-35	26	10	4	0	2	2	1
18		15	1	0	0	0	0	0
19		6	0	0	0	0	0	0
20		8	1	1	0	0	0	0
Total		55	12	5	0	2	2	1
21	Practical	42	1	2	0	0	0	0
22	Control	39	0	1	0	0	0	0
23	Diet	49	0	2	0	0	0	0
24		37	0	3	0	0	0	0
Total		177	1	8	0	0	0	0

from pullets fed diet B32. Diet B34 and the practical all-mash diet supported about the same concentration of the vitamin in the egg yolks.

*Embryonic mortality.* The classification of the embryos that died during the incubation period is shown in table 2. There was a high incidence of micromelia and syndactyly in dead embryos from pullets fed diet B31. The micromelic embryos

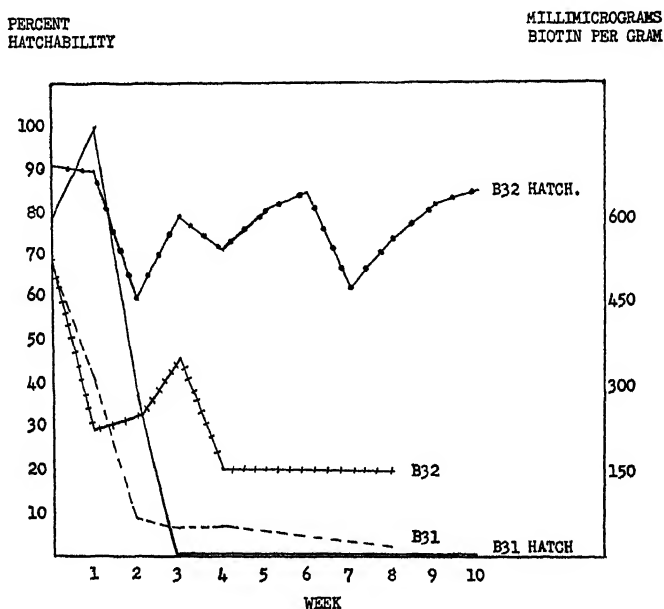


Fig. 5 Relation of carbohydrate to biotin content of egg yolk and hatchability: diet B31, sucrose; B32, dextrin.

were characterized by a parrot beak which was generally associated with a crooked tibia and a short twisted tarsometatarsus. Syndactyly and micromelia were not observed in dead embryos from pullets fed diet B32, diet B34 or from those fed the practical all-mash diet. The incidence of micromelia and syndactyly was high in the dead embryos from pullets fed diet B33 and was low in those from pullets fed diet B35, although these results are inconclusive due to the poor egg production of the birds.

## DISCUSSION OF RESULTS

It may be noted from figures 2, 3, 4 and 5 that dextrin (diet B32) maintains a higher level of biotin in the egg yolk than does sucrose (diet B31) and that this is associated with a much higher per cent hatchability. The biotin content of egg yolks from pullets fed diet B31 decreased from about 500 to 50  $\mu\text{g}$  of biotin per gram during the first 3 weeks and had decreased to about 16  $\mu\text{g}$  of the vitamin per gram by the end of the eighth week. The decrease in the per cent hatchability of eggs from pullets fed diet B31 paralleled the decrease in biotin content of the yolk (fig. 5). The per cent hatchability was 79 at the start of the experiment. This increased to 100% for some unknown reason during the first week and decreased to zero by the end of the third week. It is apparent therefore that there was an insufficient amount of biotin to support embryonic development in the eggs from pullets fed diet B31. Thus it is indicated that eggs containing 50  $\mu\text{g}$  of biotin per gram of yolk and an undetectable amount in the white will not support normal embryonic development. In the case of pullets fed diet B32 there was a decrease in the biotin content of the yolk to about 150  $\mu\text{g}$  by the end of the fourth week and this amount of biotin was found in the yolks at the end of the eighth week. Hatchability of eggs from pullets fed diet B32 was 91% at the start of the experiment and varied from 60 to 85% in the course of the 10-week period. It is realized that the per cent hatchability of eggs from pullets fed diet B32 is lower than that of pullets fed diet B34 and also lower than that of pullets fed the practical all-mash diet. Embryonic abnormalities which appeared to be identical with those previously described by Cravens et al. ('44), as occurring in eggs from hens fed a low-biotin diet, were noted in eggs from pullets fed diet B31 but did not appear in eggs of pullets fed diet B32 (table 2). From these data it is indicated that eggs which contain 150  $\mu\text{g}$  of biotin per gram of yolk on a fresh weight basis will support embryonic development even though hatchability may be somewhat reduced.

Supplementation of the sucrose diet with biotin (diet B34) resulted in a per cent hatchability and biotin content of the eggs comparable to those obtained by feeding a practical all-mash diet (figs. 2, 3 and 4). These data indicate that the deficiency which existed in the pullets fed diet B31 was a biotin deficiency.

The results obtained with dextrin (diet B32) as compared to those obtained with sucrose (diet B31) suggest that there is a marked synthesis of biotin in the intestinal tract. Further, there is good evidence that the biotin synthesized in the intestinal tract of the pullets fed dextrin was absorbed and deposited in the egg. This latter fact was established through biotin analyses of the eggs and by the fact that the biotin in the eggs was available to the embryo as evidenced by the per cent hatchability.

This is in agreement with the work of Mannering et al. ('44) in which it was reported that dextrin favored the synthesis of riboflavin in the rat, and also with the work of Teply et al. ('47) in which it was reported that dextrin diets produced the greatest synthesis of niacin and folic acid in the rat.

The data presented in this paper indicate that the laying hen is a desirable animal for studying intestinal synthesis since a determination of vitamin content of the egg offers a method of evaluating the extent of synthesis and subsequent absorption of vitamins from the intestinal tract which is difficult with other animals.

The studies reported herein shed no light on the important problem of where synthesis and absorption take place in the intestinal tract. Studies by Johansson et al. ('47) which were conducted on the birds used in the present investigation indicate that there was a marked difference in the intestinal flora of birds fed the various diets. Dextrin appeared to favor a biotin-synthesizing organism while sucrose favored a biotin-utilizing organism.

The biotin content of the eggs did not appear to be related to the egg production of the hens in the cases of pullets fed

diets B31 (sucrose) or B32 (dextrin). This is in agreement with the work of Cravens et al. ('42).

Results obtained by replacing a part of the sucrose with lactose (diet B33) or with dried whey (diet B35) are somewhat inconclusive due to the poor egg production of the pullets fed these diets. It is probably true that the level of lactose and dried whey used in these diets was too high for best results and suggests that the laying pullet may not tolerate lactose in quantities equal to that of the rat (Geyer et al., '46). Lactose, in the quantity used in diet B33, did not appear to favor the intestinal synthesis of biotin (figs. 2, 3 and 4). The biotin content and per cent hatchability of eggs from pullets fed diet B33 (lactose) followed the same general trend as those of pullets fed diet B31 (sucrose). Embryonic abnormalities, which were associated with a low biotin diet, were also observed in embryos from pullets fed diet B33 (table 2).

The results obtained with diet B35 (dried whey) were quite inconclusive after the third or fourth week due to the poor egg production of the pullets fed this diet (fig. 1). There was a higher level of biotin in the egg yolks and whites and a higher per cent hatchability of eggs from pullets fed diet B35 during the first 3 weeks than was noted in eggs from pullets fed diets B31 and B33. This may have been traceable to the fact that 25% dried whey contributed approximately 40  $\mu$ g of biotin per kilogram of diet. A small number of embryos exhibiting symptoms of a biotin deficiency were noted in the eggs of 1 pullet fed this diet (table 2).

The term "micromelia" is used in contrast to the term "chondrodystrophy" in referring to the characteristic embryonic abnormality associated with a deficiency of biotin in the diet of the mother hen. It is recognized that the term "chondrodystrophy" designates a certain histopathologic picture. It should be pointed out, however, that from a gross macroscopic study the micromelia embryos recorded in table 2 of the present report and described by Cravens et al. ('44) are indistinguishable from the chondrodystrophy which has been shown to have a hereditary basis (Lamoreaux, '42).



The occurrence of micromelia and syndactyly in embryos from pullets fed diet B31 extends and confirms the previous work of Cravens et al. ('44). It may be noted from table 2 that the characteristics which describe micromelia: parrot beak, crooked tibia and short-twisted tarsometatarsus appeared to occur in about the same frequency as syndactyly. This indicates that syndactyly is a definite symptom of biotin deficiency in the developing chick embryo as was also observed by Cravens et al. ('44). Micromelia and syndactyly were observed among embryos which lived beyond the eighth day of incubation from pullets fed diets B31 and B33. The fact that eggs from these birds were low in biotin has been discussed earlier. The incidence of micromelia and syndactyly appeared to be fairly well distributed among the individual pullets fed diets B31 and B33 (table 2).

#### SUMMARY

Evidence is presented to show that dextrin favors the intestinal synthesis of biotin in the mature fowl and sucrose does not promote the synthesis of this vitamin. The results obtained with lactose and dried whey indicate that lactose does not stimulate the synthesis of biotin in the intestinal tract of the laying pullet.

The laying hen is a desirable animal for studying intestinal synthesis. A determination of the vitamin content of the egg offers a method of evaluating the extent of synthesis and subsequent absorption of vitamins from the intestinal tract which is difficult in other animals.

There is a failure of embryonic development when the egg yolk contains as little as 50  $\mu\text{g}$  of biotin per gram on a fresh weight basis. Eggs which contain 150  $\mu\text{g}$  of biotin per gram will support normal embryonic development.

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# HUMAN UTILIZATION OF ASCORBIC ACID FROM MUSTARD GREENS

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TWO FIGURES

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Previous investigations have shown that, for many families, cooked vegetables are the main dietary source of ascorbic acid (Grigsby et al., '42; Bryson et al., '42; Coco et al., '43; Whitehead, '47). In the far South cooked greens, repeatedly shown by chemical determination to be good sources of vitamin C, are commonly used. A study of the availability for human subjects of vitamin C from such sources is therefore of practical value to the nutritionist.

A number of studies have been reported which pertain to the utilization of dietary ascorbic acid as compared to that of the crystalline vitamin. The levels at which the vitamin has been fed, the number of subjects and the duration of the test periods have varied widely. Some workers have based conclusions on urinary excretion of the ascorbic acid, while others have used plasma or whole blood levels, and a few have used both plasma or whole blood levels and urinary excretion.

After a study of the urinary excretion of ascorbic acid following the administration of comparable quantities of this vitamin as orange juice and in the pure crystalline form, Hawley, Stephens and Anderson ('36) concluded that the vitamin C of orange juice was as well utilized as the pure compound.

Baked potato was found by Clayton and Folsom ('40) to produce as high plasma ascorbic acid values as did the crystalline vitamin. Two-thirds of the dietary ascorbic acid was supplied by this vegetable.

Todhunter and Fatzer ('40) compared the utilization of ascorbic acid from red raspberries with that of the crystalline vitamin. Blood plasma ascorbic acid levels and urinary excretion of ascorbic acid were similar for the same subject, regardless of the source of the vitamin.

In 1942 Todhunter, Robbins and McIntosh reported a study of the rate of increase of blood plasma ascorbic acid after ingestion of 50 mg of ascorbic acid in the crystalline form, and after consumption of equivalent amounts in cauliflower, orange sections, orange juice or strawberries. Orange juice and orange sections produced the maximum rise in plasma level as quickly as the pure substance, while strawberries required slightly longer, and cauliflower the longest time of all the foods tested.

Using the ability of the supplement to maintain tissue saturation and to support plasma ascorbic acid levels as the criteria for judging availability, Clayton and Borden ('43) concluded that both canned tomato juice and raw cabbage produced as good results as the vitamin in tablet form.

Hartzler ('45) found no significant differences in the availability of the ascorbic acid of papayas or guava juice as compared with synthetic ascorbic acid. The criteria used for comparison of availability were the daily urinary excretion and plasma ascorbic acid levels determined once each week.

Einbecker et al. ('47) found that increases in plasma ascorbic acid produced by a test meal including frozen strawberries were comparable to those obtained when crystalline vitamin C was taken in amounts equivalent to the reduced ascorbic acid in the strawberries. Increases obtained when crystalline ascorbic acid was taken in amounts equivalent to the apparent total ascorbic acid of the strawberries were greater than those obtained after the ingestion of strawberries.

Elliott and Schuck ('47) reported approximately equal utilization of ascorbic acid from grapefruit and from the crystalline vitamin. The criteria upon which this conclusion was based were urinary excretion of ascorbic acid by 9 subjects during two 3-day test periods and whole blood ascorbic acid levels of 3 subjects at the end of each test period.

#### EXPERIMENTAL PROCEDURES

Mustard greens were chosen as the green leafy vegetable for this experiment because they are widely used in the South and a good supply was available at a convenient time.

The daily basal diet consisted of 1 egg, 1 pint of pasteurized milk, and 1 serving of each of the following: a dried fruit, a canned fruit, thoroughly cooked meat, canned snap beans, and a cooked fresh vegetable. Carrots, eggplant, potatoes, and mature dry onions were the vegetables used. The eggplant was sautéed, while the other fresh vegetables were boiled in a large amount of water to reduce the ascorbic acid content. The potatoes were always mashed. Whole grain and enriched cereals, bread, cake, cookies, and baked beans were used *ad libitum*.

During the periods when the mustard greens were eaten, the greens were substituted for the canned snap beans in the noon meal. During the periods when the synthetic vitamin was used, the ascorbic acid supplement was also given at the noon meal. Since the diet was believed to be adequate in all respects except ascorbic acid, no other vitamin supplements were given. The mustard greens were blanched and frozen before the beginning of each experiment to insure a regular supply. A pressure saucepan was used for cooking as previous experience (Hollinger, '44; Hollinger and Colvin, '45) had shown that this method of cooking results in maximum retention of vitamin C. The greens were seasoned with sufficient salt and bacon fat before cooking to make them palatable.

In 1945, 5 young women served as subjects. The plan of the experimental periods is given below.

*Experimental periods — 1945*

*Period I, 14 days.* Basal diet containing 13 mg of reduced ascorbic acid.

*Period II, 21 days.* Basal diet supplemented by 125 gm of mustard greens at the noon meal in place of canned snap beans. The average ascorbic acid content of this diet was 57 mg, of which 49 mg were supplied by the greens.

*Period III, 14 days.* Basal diet as in Period I with supplement of ascorbic acid in water solution to give same average total intake as in Period II.

Immediately after breakfast blood samples were drawn by venous puncture on 5 consecutive days each week. Analyses were made immediately by the Mindlin and Butler ('38) macro method. The method was followed in detail except that no potassium cyanide was used.

All foods containing ascorbic acid were eaten in constant weighed amounts and a determination of the reduced ascorbic acid was made daily on a composite sample representing one-fourth of the intake. Determination of ascorbic acid in the food was made by the dye titration method.

In 1946 the plan of the experiment was altered in order to provide for a period of high ascorbic acid intake immediately preceding the beginning of the experimental period and again following the period when mustard greens were eaten. The plan of the experimental period is given below. This plan insured a more uniform state of nutrition among the subjects than is probable when no preparation of the subjects is carried out.

*Experimental periods — 1946*

*Period I, 15 days.* Uncontrolled diet. One hundred milligrams ascorbic acid daily in tablet form.

*Period II, 14 days.* Basal diet containing an average of 15 mg of ascorbic acid.

*Period III, 15 days.* Basal diet supplemented by 125 gm of mustard greens at the noon meal in place of canned snap

beans. The ascorbic acid content of this diet was 57 mg, of which 49 were supplied by the cooked greens.

*Period IV, 15 days.* Same as Period I.

*Period V, 14 days.* Same as Period II.

*Period VI, 15 days.* Basal diet supplemented by sufficient ascorbic acid in solution to bring the average total intake of the vitamin to that in Period III.

Blood samples were drawn immediately after breakfast 4 times each week. The samples were taken on Monday, Tuesday, Thursday and Friday in order to compare values on consecutive days. Eight samples from each subject were analyzed during the depletion periods (Periods II and IV) and 10 during the periods when the greens and the ascorbic acid were given (Periods III and VI).

Three young men and 4 young women served as subjects for this portion of the study. Three of the young women (subjects J.G., L.J., and V.W.) had served as subjects in 1945.

## RESULTS

In 1945 the average plasma ascorbic acid values for 3 subjects were almost the same on both regimes. For 1 subject the average plasma ascorbic acid value was 14% higher on the ascorbic acid regime than on the mustard green regime, while in another the crystalline vitamin produced an average plasma ascorbic acid value 33% higher than that produced by mustard greens (table 1). In 1946, 6 subjects showed slight increases in plasma ascorbic acid values during the mustard green regime when these values were compared to the average for the 2 days just previous. One subject had the same average plasma value (table 2). During the period when ascorbic acid was taken, 4 subjects showed gains while the remaining 3 showed slight losses. Subject V.W. showed considerably greater gain on crystalline ascorbic acid than on mustard greens. It should be noted that this subject had served in the 1945 study and at that time also attained higher plasma ascorbic acid values when receiving the pure vitamin. Subjects



J.G. and L.J. who also had served in the 1945 study showed in both experiments approximately the same plasma ascorbic acid values on the ascorbic acid regime as on the mustard greens diet.

TABLE 1

*Average plasma ascorbic acid values produced by mustard greens and by crystalline ascorbic acid (in milligrams per cent) 1945.*

SUBJECT	SEX AND WT. IN KG	PERIOD I BASAL DIET <sup>1</sup>	PERIOD II MUSTARD GREENS <sup>2</sup>	PERIOD III ASCORBIC ACID <sup>3</sup>
D. C.	F. 57	0.50	0.46	0.48
J. G.	F. 68	0.67	0.53	0.53
L. J.	F. 66	0.30	0.48	0.51
H. L.	F. 57	0.45	0.58	0.66
V. W.	F. 50	0.58	0.58	0.76
Average		0.50	0.53	0.59

<sup>1</sup> Average of 2 days preceding mustard green period.

<sup>2</sup> Average of 15 determinations during 3 weeks.

<sup>3</sup> Average of 10 determinations during 2 weeks.

TABLE 2

*Average plasma ascorbic acid values produced by mustard greens and by crystalline ascorbic acid (in milligrams per cent) 1946.*

SUBJECT	SEX AND WT. IN KG	AT END OF PERIOD II <sup>1</sup>	MUSTARD GREENS <sup>2</sup>	AT END OF PERIOD V <sup>1</sup>	CRYSTALLINE ASCORBIC ACID <sup>2</sup>
J. B.	M. 60	0.39	0.46	0.52	0.51
J. G.	F. 68	0.47	0.52	0.48	0.51
V. H.	F. 60	0.56	0.57	0.54	0.61
L. J.	F. 66	0.45	0.45	0.50	0.49
F. L.	M. 72	0.29	0.36	0.50	0.43
J. W.	M. 76	0.36	0.39	0.36	0.39
V. W.	F. 50	0.52	0.54	0.50	0.63
Average		0.44	0.47	0.48	0.51

<sup>1</sup> Average of plasma values for last 2 days of period.

<sup>2</sup> Average of plasma values (10 determinations during 2 weeks).

Higher plasma values were maintained at all times by the smaller subjects while the larger subjects showed a more rapid decline on the basal diet and a very slow decline on both the mustard green and the crystalline ascorbic acid regime.

This suggests that the average daily intake of ascorbic acid, 59 mg, was inadequate for the larger individuals. Typical curves are shown in figures 1 and 2. Plasma values for the men showed fluctuations similar to those for the women.

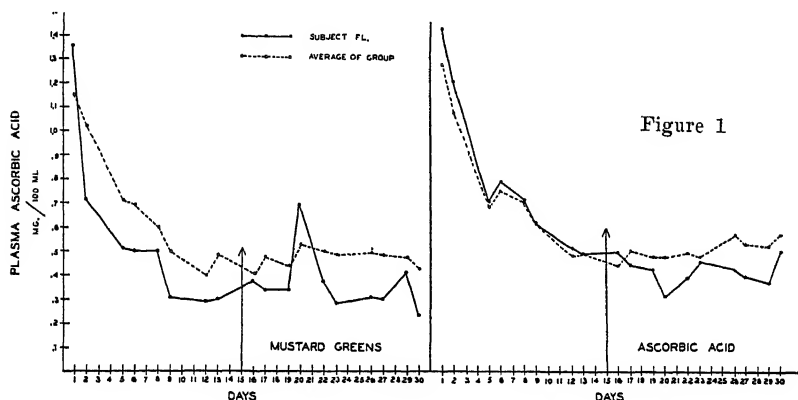


Figure 1

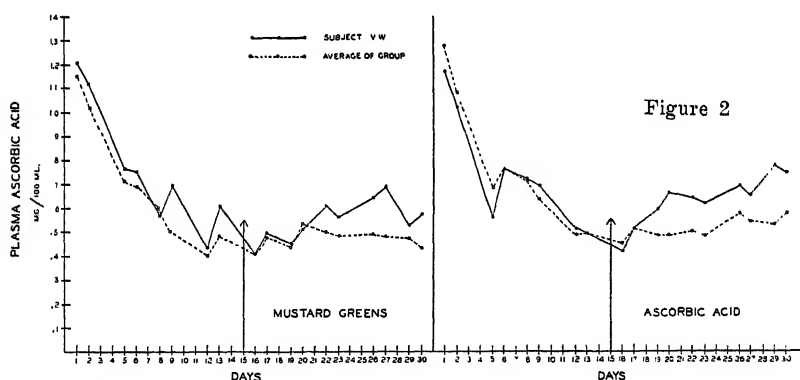


Figure 2

Figs. 1 and 2 Comparison of plasma ascorbic acid values of subjects F.L. and V.W. and average plasma ascorbic acid values of 7 individuals — 1946.

### CONCLUSION

It is concluded that some individuals utilize the ascorbic acid from cooked mustard greens just as well as the synthetic vitamin. Other persons fail to show as high plasma ascorbic acid values when mustard greens are the main source of the vitamin as they do when the crystalline vitamin is given.

## ACKNOWLEDGMENTS

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# FURTHER EXPERIMENTS ON THE RELATION OF FAT TO ECONOMY OF FOOD UTILIZATION

## III. LOW PROTEIN INTAKE <sup>1</sup>

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Two recent publications in this journal by Forbes, Swift and associates ('46a, '46b) demonstrated that liberal amounts of fat in the diet of the growing rat resulted in a superior energy utilization of the diet as a whole. Increased body weight, comprising greater gains of fat and energy, and a decrease in the heat production of growing rats resulted as the fat content of isocaloric diets was increased from 2 to 30%. In the preparation of the diets used in these experiments an effort was made to furnish all nutrients in optimum quantities for the most efficient utilization of the food energy, including an excellent quality protein mixture furnishing 22% protein in the diet.

In view of the shortages of foodstuffs current in many areas of the world, especially of fat and protein, it seemed of further interest to investigate the effect of decreasing the dietary protein in similar high and low fat diets on the resulting energy utilization. It was decided, therefore, to follow the plan of the earlier experiments, namely, to use diets containing 2 and 30% of fat, but with a protein content of 7% in the low fat diet and an equivalent amount in the high fat diet.

<sup>1</sup> Authorized for publication on September 15, 1947, as paper no. 1391 in the Journal Series of the Pennsylvania Agricultural Experiment Station.

## EXPERIMENTAL

The experimental subjects were 12 pairs of litter-mate male albino rats of the Wistar strain selected immediately after weaning. Each rat of a pair was fed isocaloric amounts of one of the 2 diets for a period of 70 days, using the paired-feeding technique. Both rations supplied the same amount of protein and vitamin supplements, and differed inversely in their fat and carbohydrate contents.

The subjects were allowed normal cage activity. The heat production as determined by the body balance method of this laboratory (Swift et al., '34) provided a single measurement for the entire 70-day period through the subtraction of the energy of the excreta and of the body gain from the gross energy of the food.

Daily feces and urine collections were made according to the method referred to above and all energy values of the diets, bodies, and excreta were determined by the bomb calorimeter. Nitrogen and crude fat determinations were made according to the Kjeldahl and Soxhlet extraction procedures, respectively.

The 2 diets, prepared from purified foodstuffs in the manner described earlier, were analyzed before the start of the experiment and stored in the refrigerator. Tables 1 and 2 present the composition of the diets and the quantities of major nutrients supplied.

The average weekly live weights for the 12 rats on each of the 2 diets are shown in table 3. It is apparent that the animals on the high fat diet gained more weight than the low fat group as the experiment progressed. The statistical odds that this difference in weight gain is significant are 50:1. The difference in live weight is further emphasized, however, by the method of food assignment used which has the effect of placing at a disadvantage those animals that increase more rapidly in weight since a larger proportion of the food intake is then required for maintenance and less is available for growth.

The possibility that this increased weight gain could be in the form of water is contraindicated by the data on the body

TABLE 1  
*General composition of diets.*

COMPONENTS	DIET 1	DIET 2
	%	%
Protein-containing mixture <sup>1</sup>	11.00	15.63
Carbohydrate mixture <sup>2</sup>	83.00	50.37
Corn oil <sup>3</sup>	2.00	2.00
Lard <sup>4</sup>	0.00	28.00
Salt mixture <sup>5</sup>	4.00	4.00
Cal. per gm	3.869	5.498
Iso-caloric factors	1.000	0.704

<sup>1</sup> Casein 50%, skim milk powder 25%, irradiated yeast 25%. Mixture contained 60.5% protein ( $N \times 6.25$ ) and 4.71 cal. per gm.

<sup>2</sup> Corn starch 34%, sucrose 33%, dextrin 12%, and dextrose (cerelose) 21%. Mixture contained 3.75 cal. per gm.

<sup>3</sup> Mazola. Contained 9.52 cal. per gm.

<sup>4</sup> Contained 9.33 cal. per gm.

<sup>5</sup> U.S.P. XII. no. 2.

TABLE 2  
*Vitamins added per kg of diet 1 and to iso-caloric quantities of diet 2.*

VITAMINS		VITAMINS	
	mg		mg
Carotene	40	Choline chloride	2000
Thiamine hydrochloride	20	Alpha tocopherol	200
Riboflavin	20	P-aminobenzoic acid	200
Pyridoxine hydrochloride	20	2-methyl-1, 4 naphtho-	
Niacin	20	quinone	6
Calcium pantothenate	100	Inositol	2000

TABLE 3  
*Average live weights of rats during 10 weeks on iso-caloric quantities of diets containing 2% and 30% of fat.*

FAT CONTENT OF DIETS	INITIAL BODY WEIGHT	WEEK NUMBER									
		1	2	3	4	5	6	7	8	9	10
%	gm	gm	gm	gm	gm	gm	gm	gm	gm	gm	gm
2	54	58	70	81	89	97	105	113	123	129	135
30	54	58	71	83	91	99	107	116	126	133	140



gain of fat which are summarized in table 4. The rats on the high fat diet gained, on an average, 14% more energy in the form of fat than the group on the low fat diet. The statistical odds that this difference is not due to chance are 25:1.

The average gain of ether extract in the rats on the 2% fat diet was 19.4 gm, while their average intake was only 11.5 gm.

TABLE 4

*Average food consumption during 70 days; and initial and final nitrogen and fat contents of rat bodies.*

FAT CONTENT OF DIETS	FOOD CONSUMPTION	NITROGEN INTAKE	BODY NITROGEN		BODY FAT	
			Initial	Final	Initial	Final
%	gm	gm	gm	gm	gm	gm
2	576.4	6.48	1.30	3.85	4.13	19.36
30	405.8	6.31	1.30	3.86	4.13	21.67

TABLE 5

*Partition of average nitrogen intake per rat during 70 days.*

FAT CONTENT OF DIETS	INTAKE OF N	OUTPUT OF N IN		N RETAINED <sup>1</sup>	N RETAINED <sup>2</sup>
		Urine	Feces		
%	gm	gm	gm	gm	gm
2	6.48	2.89	0.85	2.74	2.55
30	6.31	3.04	0.71	2.56	2.56

<sup>1</sup> Nitrogen in food minus nitrogen in the excreta.

<sup>2</sup> Nitrogen in bodies of rats fed for 70 days minus nitrogen in the bodies of the control group.

Therefore there was an average of nearly 8 gm of fat synthesized by the rats on this diet. The ether extraction procedure was employed to separate the body residue into 2 portions for the purpose of quantitative grinding and sampling. No effort was made to determine precisely the true fat gains since the weights of body fat were not of primary significance.

A summary of the nitrogen utilization data and the partition of the nitrogen intake are presented in table 5. The reduction in protein content of these diets in comparison with the earlier work resulted in a considerably decreased growth rate and nitrogen retention. However, the difference in fat

content of the diets was found to exert no significant influence on the nitrogen retention. This finding is in accord with that of the former work which indicated that the influence of large amounts of fat in the diet on the nitrogen utilization was not very definite.

The recovery of the feed nitrogen in the feces, urine, and body gain was, on the average, 97 and 100% of the intake for the 2% and 30% fat diets, respectively. The comparison of nitrogen retained as calculated from intake and excretion, and the nitrogen of the body gain were in close agreement.

From the data in table 6 it is evident that the difference in fat content of the diets resulted in a significant difference in heat production. The high fat animals produced 2.4% less

TABLE 6

*Partition of average daily intake of food energy per rat during 70 days.*

FAT CONTENT OF DIETS	GROSS ENERGY INTAKE	ENERGY INTAKE				ENERGY OUTPUT			ENERGY RETAINED
		Protein	Carbo- hydrate	Fat	Metabo- lizable	In feces	In urine	As heat	
%	Cal.	Cal.	Cal.	Cal.	Cal.	Cal.	Cal.	Cal.	Cal.
2	2230	233	1886	111	2132	62	37	1894	238
30	2230	226	837	1167	2112	88	31	1849	263

heat than the pair-mates on the low fat diet. The odds that this difference is not due to chance are 280:1.

Slightly more energy was excreted in the feces and less in the urine of the animals on the high fat diet as compared to the low fat group but the resulting metabolizable energy values were essentially the same.

In general, the effect of reducing the protein content of the diets from about 22%, as fed to growing rats in the previous work, to 7% in the present study was mainly in degree rather than in nature. Despite the lower protein content of the diets the high fat ration exerted a favorable effect upon the energy utilization, demonstrated by an increase in body gain and a decrease in heat production. It seems probable from this evidence that the level of dietary protein is not a factor in the

superior energy utilization of high fat diets exhibited by growing albino rats under conditions of normal cage activity.

#### SUMMARY

An investigation of the possible influence of low protein intake on the superior energy utilization of high fat diets was conducted using 12 litter-mate pairs of weanling male albino rats subjected to a 70-day metabolism and body analysis procedure.

A comparison was made between 2 diets containing 2% and 30% of fat, respectively, so prepared and fed as to supply to each rat of a litter-pair and therefore to each group of 12 the same quantities of energy, protein and vitamins.

Determinations were made of gains in live weight, nitrogen, fat and energy, and of the heat production for 70 days as the energy of the food minus the energy of the excreta and of the body gain.

Decreasing the protein intake from the former 22% level to 7% of the diet did not alter the previously reported superior energy utilization of high fat diets by the growing rat. In the present experiment an increased weight gain, increased body gains of fat and energy, and decreased heat production were associated with the high fat ration.

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# EFFECT OF THE PTEROYLGLUTAMIC ACID INTAKE ON THE PERFORMANCE OF TURKEYS AND CHICKENS<sup>1</sup>

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Previous work has shown that the young turkey requires a dietary source of pteroylglutamic acid (folic acid) and that a deficiency of this factor in the diet results in retarded growth, moderate anemia, cervical paralysis and a high mortality (Richardson et al., '45; Jukes et al., '47; Russell and Taylor, '47). Available data indicate that 0.8–2.0 mg of pteroylglutamic acid/kg of ration is required for the young poult (Jukes et al., '47; Russell and Taylor, '47).

In a recent report by Taylor ('47) data have been presented on the influence of the amount of pteroylglutamic acid (PGA) in the diet of the White Leghorn on egg production and hatchability. It was concluded that 0.12 mg/kg of PGA or less was required for high egg production but that more than this amount was needed for sustained hatchability. No work has been reported, however, on the effect of the amount of this vitamin in the diet on the performance of adult turkeys for egg production or hatchability.

In the present study the influence of the amount of PGA in the diet on the performance of adult turkeys and chickens and of young poults has been determined. Data have also been

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obtained during the past 2 seasons on the amount of PGA found in the egg and in the blood of both chickens and turkeys as influenced by the dietary intake, the survival and performance of young poults obtained from groups of hens fed diets high and low in PGA, and egg production and hatchability. The results obtained in these experiments are reported in this paper.

#### EXPERIMENTAL

*Care of experimental birds.* Broad-breasted, bronze turkey hens that were 6 months of age were placed in breeding pens at an average initial weight of approximately 7.7 kg. Eight birds were used in each of 2 groups; 1 received a diet designed to be low in PGA and the other received the same diet to which 2.0 mg PGA/kg were added. Insofar as possible the birds were distributed uniformly between the 2 groups. The houses were lighted at all times and good egg production was achieved in a period of 2 weeks. The hens were then trap-nested and records obtained on egg production. At uniform intervals throughout the experiment studies were also conducted on the hatchability of the eggs and on the amount of PGA in the egg. A similar allocation of experimental birds was used in each of the 2 seasons that this study was carried out.

First-year White Leghorn pullets (hereafter referred to as hens for simplicity) were used in parallel investigations. Fifteen hens were used in each group, 1 group receiving the basal ration and the other the PGA supplemented ration. In all cases matings were carried out with males maintained on a standard ration and insofar as possible the males were rotated among several groups of hens at regular intervals.

A series of studies was also carried out with young poults in which the effect was determined of the amount of PGA ingested by the hens on the survival and growth of the young poults fed diets deficient or adequate in the vitamin. In this work day-old poults both from the deficient and supplemented hens were used. Poults hatched from eggs laid by each hen were distributed uniformly among the experimental groups.

*Composition of rations.* The composition of the rations fed the adult turkeys is given in table 1. The basal ration was designed to be low in PGA and adequate insofar as possible in other nutrients. The control group was fed, in addition, 2.0 mg of PGA/kg of ration. The B vitamin supplements were prepared in the following manner. An aqueous suspension of the vitamins was added to finely ground corn, dried, reground to a fine powder and thoroughly mixed with additional ground corn so that 1% of the mixture supplied the correct amounts of the vitamins in the ration. The adult chickens were fed a similar ration, differing only in that 2% less casein was used with a corresponding increase in the amount of cerelese.

In preliminary tests conducted during 1946 a ration similar to that just described, except higher in protein, was used in studies with the young poult. In 1947, however, a purified ration of known PGA content was used. The effect of the diet of the hen on the performance of the young poult could, therefore, be more accurately evaluated. The composition of the purified ration used in this work with the young poults is also given in table 1. The amount of PGA added to the ration was varied, being 0.2, 0.8 or 2.0 mg/kg. These levels were used in order to determine the effect of feeding amounts either below, approximating or exceeding the reported requirement.

*Methods of analysis.* The pteroylglutamic acid content of the eggs was determined microbiologically with the use of *S. faecalis* R as the test organism (Teply and Elvehjem, '45). The vitamin was liberated from the samples by takadiastase treatment in a manner similar to that used for animal tissues (Schweigert et al., '46). The entire content of each egg was weighed and homogenized in a Waring blender with an equal weight of water. An aliquot of the homogenate (equivalent to 5 gm of egg) was then diluted to approximately 75 ml, adjusted to pH 4.5 with HCl and sodium acetate, and incubated with 100 mg of takadiastase for 16-18 hours at 37°C. After autoclaving for 5 minutes to inactivate the enzymes, the samples were diluted to an appropriate volume, neutralized, filtered and aliquots taken for assay.

TABLE 1  
*Composition of rations.*

RATIONS FOR ADULT TURKEYS			RATIONS FOR TURKEY POULTS			
	%	mg/kg		%	mg/kg <sup>1</sup>	
Ground yellow corn	65.54		Casein	25	Thiamine	6.0
Casein	10	6.6	Gelatin	10	Riboflavin	6.6
Raw bone meal	8	6.6	Corn oil	4.25	Pyridoxine	6.6
Cerelose	11.4	22	Salts <sup>2</sup>	5.0	Ca pantothenate	22
Cellulose	2	50	Fortified A and D oil	0.75	Nicotinic acid	50
Oyster shell	2		Cystine	0.2	Inositol	1000
NaCl	0.6		Starch	54.8	Choline	2000
MnSo <sub>4</sub> · 4H <sub>2</sub> O	0.013	0 or 2.0			p-aminobenzoic acid	2.0
Choline	0.17				Biotin	0.2
Fortified A and D oil	0.25				2-methyl 1,4 naphthoquinone	20
					Mixed tocopherols	100
Delsterol	0.026				Pteroylglutamic acid	0.2, 0.8 or 2.0

<sup>1</sup> Wilkening and Schweigert ('47).

<sup>2</sup> Hegsted et al. ('41).

A series of determinations conducted during the 1946 season indicated that the amount of PGA added to the diet did affect the amount in the eggs. Therefore, during the present season, 8-15 eggs were taken from each group of chickens and turkeys at regular monthly intervals for PGA analysis.

At the conclusion of the feeding tests conducted with adult chickens and turkeys, samples of blood were drawn for determinations of free and combined PGA (Schweigert and Pearson, '47). The feed was withheld from the birds for 3 hours prior to taking of the blood samples. Six to 8 ml of blood were drawn from the turkeys from the wing vein and from the chickens by heart puncture into oxalated tubes. Determinations were made of the "apparent" free folic acid in whole blood and plasma, and in some cases of the amount present after enzymatic digestion on both plasma and whole blood.

#### RESULTS AND DISCUSSION

No effect attributable to feeding the diet low in PGA to adult turkeys or chickens was observed on general appearance, mortality, hemoglobin level, or weight change. Furthermore, no appreciable difference was noted in egg production and hatchability for the 2 species. The egg production for the group of 8 turkeys fed the diet low in PGA totalled 1108 and the egg production for the supplemented group totalled 1067 for the 2 seasons. Egg production for each group of 15 chickens fed the 2 corresponding diets was 1928 and 1945, respectively. Thus, it appears that sufficient PGA was present in the basal diet as judged by these criteria. By microbiological analysis, the basal ration was shown to contain 0.42 mg of PGA/kg of ration.<sup>2</sup>

It is significant to note that Taylor ('47) observed that 0.12 mg of PGA/kg was sufficient for satisfactory egg production and somewhat more was needed for hatchability. It would seem, therefore, that the requirement for good hatchability was between 0.12 and 0.42 mg of PGA/kg.

<sup>2</sup> We are indebted to Dr. T. H. Jukes and Dr. E. L. R. Stokstad for this analysis. A chick pancreas enzyme preparation was used to liberate the PGA.



The level of PGA in the diet does, however, markedly influence the amount of the vitamin present in the egg. Preliminary results were obtained on eggs from turkeys maintained for 5-6 months on the experimental regimen described and those from chickens maintained for periods of 1-2 months during the 1946 season. Twenty turkey eggs from the low PGA group averaged  $0.113\text{ }\mu\text{g}$  of PGA/gm; 26 eggs from the PGA supplemented group averaged  $0.195\text{ }\mu\text{g}$  of PGA/gm, while 13 eggs from hens fed the standard flock ration averaged  $0.171\text{ }\mu\text{g}$  of PGA/gm. Separate studies showed that most of the vitamin was present in the egg yolk:  $0.47$  and  $0.31\text{ }\mu\text{g}$  of PGA/gm of yolk for the supplemented and deficient groups, respectively; and  $0.013$  and  $0.007\text{ }\mu\text{g}$  of PGA/gm of egg white, respectively.

Eggs from chickens fed the basal and supplemented diets for 1 month were identical in their PGA content:  $0.067$  and  $0.064\text{ }\mu\text{g/gm}$  of whole egg; however, eggs obtained after 2 months of feeding averaged  $0.12$  and  $0.20\text{ }\mu\text{g}$  of PGA/gm, respectively.

Experiments conducted during 1947 were designed to obtain eggs from each group at regular intervals from the beginning to  $7\frac{1}{2}$  months (table 2). Unfortunately, egg production was not at a high enough rate to merit analyses prior to  $1\frac{1}{2}$  months on experiment. It will be noted that a difference was obtained in the content of PGA in  $1\frac{1}{2}$  months of feeding. The difference in the amount found in the eggs did not appear to become greater during the test period. Thus the mean difference was as great at  $2\frac{1}{2}$  or  $3\frac{1}{2}$  months of feeding as it was at  $6\frac{1}{2}$  or  $7\frac{1}{2}$  months. Insofar as possible, determinations were made on eggs from each hen each month; therefore each figure is the mean obtained for determinations on 8-15 eggs. The number in each group was regarded as insufficient for a separate statistical analysis; therefore the results for the entire period were analyzed statistically. It was shown that the effect of the level of PGA ingested on the amount found in the eggs of both chickens and turkeys was highly significant.

It is recognized that the techniques used for the liberation of PGA from the eggs may not have resulted in complete liberation of the vitamin. Some studies were conducted with the use of chick pancreas enzyme<sup>3</sup> or a hog kidney preparation (Bird et al., '46). Higher values were obtained with the use of these preparations in some cases; however the relative difference in the values for the eggs from the supplemented groups as compared to those from the low PGA group was

TABLE 2

*Effect of the amount of pteroylglutamic acid ingested by turkeys and chickens on the amount of this vitamin found in the egg (1947 season).*

LENGTH OF TIME ON EXPERIMENT	TURKEYS		CHICKENS	
	Basal ration	PGA supple- mented ration	Basal ration	PGA supple- mented ration
<i>Months</i>	$\mu\text{g PGA/gm of whole egg}$	$\mu\text{g PGA/gm of whole egg}$	$\mu\text{g PGA/gm of whole egg}$	$\mu\text{g PGA/gm of whole egg}$
1½	0.13	0.19	0.08	0.11
2½	0.09	0.15	0.09	0.16
3½	0.09	0.12	0.08	0.12
4½	0.16	0.30	0.15	0.17
5½	0.16	0.30	0.08	0.12
6½	0.06	0.18	0.11	0.13
7½			0.11	0.14
Total no. of eggs	44	43	59	59
Mean and S.E.	0.117 ± .007	0.209 ± .013	0.098 ± .004	0.133 ± .005
F Value	42.9 <sup>1</sup>		39.7 <sup>2</sup>	

<sup>1</sup> F Value required for significance at the 1% level is 6.93.

<sup>2</sup> F Value required for significance at the 1% level is 6.86.

consistent with the results obtained with takadiastase treatment. Therefore, in order to obtain comparable data during the experiment, the takadiastase technique was used throughout, and since equal numbers of eggs from each group of birds were included in each series of determinations, the relative values obtained are assumed to be valid. Variations in the amount found for any 1 month may be due in part to seasonal effects attributable to the level of egg production, feed con-

<sup>3</sup> Dr. L. R. Richardson generously supplied this preparation.

sumption, etc., but the figures may also represent variations in the amount of the vitamin liberated in the specific series of analyses carried out.

*Blood studies.* Analyses obtained on the "apparent" free PGA content of whole blood and plasma also demonstrated the influence of the dietary treatment. Results were obtained at the end of the experiments for both seasons on adult chickens and turkeys. In some cases, blood was taken from the same

TABLE 3

*Effect of the amount of pteroylglutamic acid ingested by turkeys and chickens on the amount of "apparent" free pteroylglutamic acid found in the blood.*

LENGTH OF TIME ON EXPERIMENT	SAMPLE USED	MILLIMICROGRAMS OF PGA/ML <sup>1</sup>		F VALUE	
		Basal ration	PGA supple- mented ration	Ob- served	Re- quired (1% level)
Turkeys					
1946					
6 months	whole blood	9.0(12)	16.8(12)	17.8	7.88
	plasma	1.7(8)	6.0(8)	127.5	8.68
1947					
7 months	whole blood	8.4(16)	15.9(16)	19.2	7.53
	plasma	2.5(16)	7.4(16)	25.1	7.53
Chickens					
1946					
2½ months	whole blood	10.2(14)	11.0(14)	not significant	
	plasma	3.2(13)	4.8(13)	13.0	7.77
1947					
7½ months	whole blood	9.7(8)	8.3(10)	not significant	
	plasma	3.8(8)	4.5(9)	not significant	

<sup>1</sup> The number of analyses is given in parentheses.

birds 2 weeks prior to the end of the experiment. These results are presented in table 3. The amount of "apparent" free PGA in the whole blood of the turkeys, for example, is significantly lower for the group fed the basal ration (8.6 µg/ml) than for the group fed the supplemented ration (16.2 µg/ml). In some cases the amount of free PGA in the cells and plasma for the 2 groups was determined. An average of 27.8 and 41.4 µg of PGA per milliliter of cells from the defici-

ent and supplemented groups, respectively was found; 1.0 and 6.0  $\mu\text{g}$  of PGA per milliliter of plasma, respectively.

No difference was noted in the values for whole blood of the chickens between the 2 groups. The values for plasma reflected the influence of the dietary treatment in tests conducted during 1946, but no significant difference was noted in those conducted in 1947. More data are needed to explain this apparent discrepancy; however uncontrolled variables such as egg production, feed consumption and time of the year may have exerted an effect. Nevertheless, the data do demonstrate that the level of the microbiologically available vitamin in the blood of the chicken is relatively insensitive to the amounts of PGA fed, as compared to that available in the turkey. The values for the supplemented groups obtained in the present study are in excellent agreement with those obtained in previous work on the "apparent" free folic acid content of blood from various species (Schweigert and Pearson, '47). The higher values observed for the amount of the free vitamin in the blood of the avian species as compared to those obtained for others may be correlated with the higher requirement of the avian species for this vitamin.

Analyses obtained after enzymatic digestion were similar for whole blood and plasma for the 2 experimental groups. The following values were obtained for the amount of PGA in whole blood after enzymatic treatment for the basal and PGA supplemented turkeys: 56.1  $\mu\text{g}/\text{ml}$  (average of 16 determinations) and 64.7  $\mu\text{g}/\text{ml}$  (average of 16 determinations), respectively. For plasma, 57.9 and 57.8  $\mu\text{g}/\text{ml}$  were observed, respectively, for the 2 groups (14 analyses in each case). The amounts observed in chicken blood were as follows: 53.8 and 55.5  $\mu\text{g}/\text{ml}$  of whole blood for the basal and supplemented groups (average of 20 determinations in each case) and 38.9 and 36.1  $\mu\text{g}/\text{ml}$  of plasma, respectively (average of 14 analyses in each case). These rather surprising observations may be of great importance. It may be possible that animals that are very deficient in this vitamin may have sufficient amounts of the conjugated form (presumably that incre-

ment measured after enzymatic digestion) present in the blood and other tissues and are unable to break it down to the biologically active or free form. Work along this line will be conducted to determine the significance of this preliminary observation. From the data obtained on whole blood and plasma, it is apparent that most of the free vitamin occurs in the cells, while the amount of PGA observed after enzymatic digestion appears to be the same for both plasma and cells.

The greater differences noted in the PGA content of the eggs and blood for the 2 groups of turkeys as compared to the 2 groups of chickens suggest that the requirement of this vitamin is greater for the turkey than for the chicken.

*Studies with the young poults.* Preliminary experiments conducted in 1946 indicated that poults from hens receiving the low PGA diet grew at a slower rate than the poults from the adequately supplemented hens when the poults were fed rations similar to those fed the hens. In another experiment, a low PGA diet, high PGA diet and the standard flock ration were fed to 10 poults in each group. These poults were from hens fed an adequate ration. At about 4 weeks of age all birds in the low PGA group became infected with fowl pox, while those in the other 2 groups (located above and below the basal group in the brooders) did not become affected. Since this disease is considered very contagious, it seemed rather surprising that only the low PGA group was affected unless the type of ration fed influences the susceptibility. Much more work with carefully controlled conditions will be needed to verify this observation.

Poults hatched from eggs obtained after the breeding turkeys had been on experiment for 4, 5 and 6 months during 1947 were used to determine the effect of the dietary intake of the hens on the performance of the young poult (table 4). Purified rations were used in which the PGA content was varied from 0.2–2.0 mg/kg for the various groups (table 1). In the first experiment poults from hens that received either the basal ration or supplemented ration were used. All 9 poults from hens receiving the former ration died within 2 weeks when

fed 0.2 mg of PGA/kg of ration. However, all poult fed 2.0 mg/kg, regardless of the diet fed the hens, lived and grew at a normal rate. Three of 5 poult from hens fed the supplemented diet died when 0.2 mg of PGA/kg was fed and the remaining 2 poult grew normally. These observations were extended in 2 subsequent experiments in which groups were fed 0.8 mg of PGA/kg of ration in addition to groups fed 0.2 or 2.0 mg/kg. These results are summarized in table 4. It

TABLE 4

*Effect of the amount of pteroylglutamic acid in the diet of the turkey hen and poult on the performance of the young poult.*

EXP. NO.	PGA ADDED TO DIET		NO. OF POULTS	WEIGHT OF POULTS				NO. OF DEATHS <sup>1</sup>	NO. WITH PEROSIS
	hens	poult		1 day	1 wk.	2 wks.	3 wks.		
	<i>mg/kg</i>	<i>mg/kg</i>		<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>		
2	none	0.2	18	59	77	124	194	8	4
	2.0	0.2	7	57	78	145	234	none	2
	none	0.8	15	56	72	125	222	3	6
	2.0	0.8	6	58	89	173	285	none	2
	none	2.0	16	59	74	125	213	1	7
	2.0	2.0	7	61	81	146	250	1	1
3	none	0.2	6	56	85	125	199	2	1
	2.0	0.2	5	53	89	159	292	none	3
	none	0.8	6	54	89	225	332	none	2
	2.0	0.8	5	52	89	213	311	none	none
	none	2.0	5	59	84	149	298	none	none
	2.0	2.0	4	54	84	165	327	none	none

<sup>1</sup> Only those poult that died after 4 or more days on experiment are included in this tabulation.

will be noted that the mortality and also the rate of growth of the young poult were influenced by the diet of the hens from which the poult were obtained. From these experiments it can be seen that a total of 10 out of 24 poult died prior to 3 weeks of age when both the hens and poult received the diets low in PGA. However, either when the hens received the PGA supplemented diet or the poult received 2.0 mg of PGA/kg of ration, few losses occurred (2 poult out of 55). The mortality observed when 0.8 mg of PGA/kg of ration was fed to the poult was intermediate when the hens had been

fed the basal ration (3 out of 19). As indicated above, an excellent performance was noted when 0.8 mg of PGA/kg was fed and the hens had been fed the supplemented ration. The rates of growth observed also reflected the dietary treatment of the hens as well as that of the poults. The requirement of the poults appeared to be between 0.8 and 2.0 mg/kg of PGA judged on the basis of rates of growth and mortality when the hens had been fed diets low in this vitamin. It appears that 0.8 mg of PGA/kg is adequate for the poults when the hens had been fed a diet to which 2.0 mg of PGA had been added. Thus, these results may form a basis for explaining the differences in the requirements for PGA reported by Jukes and associates ('47) and Russell and Taylor ('47). The present work suggests that differences in the PGA nutrition of the hens may be a contributing factor in determining the amount required by the young poult.

Three of the poults from the hens fed the low PGA diet developed typical cervical paralysis (Richardson et al., '45; Jukes et al., '47) at 17-19 days of age when receiving 0.2 mg of PGA/kg. No other groups demonstrated this syndrome. The incidence of perosis was also higher for the poults from hens receiving the diet low in PGA, particularly in the second experiment. Thus, it has been demonstrated that feeding diets low in PGA to the hen adversely affects the ability of the young poult to survive and grow, and also increases the amount of the vitamin needed in the diet of the poult for normal development. These observations suggest that some of the mortality observed in young poults is due to subminimal levels of various nutrients, including PGA, present in the diet of the hens. Perhaps more attention should be focused on the development of improved rations for the breeding flock as well as for the growing birds.

Although no effect could be demonstrated on the egg production or hatchability due to ingestion by the hens of a diet low in PGA, the use of other criteria, namely, the levels of PGA in the eggs and blood and the performance of young poults from these hens suggests that the level of PGA fed

(0.42/kg of ration) was inadequate for the breeding turkey. The use of several criteria, therefore, in evaluating the nutritive value of the diet is highly desirable. With improvements in methods for liberating this vitamin from natural materials, more exact information on its distribution in feeds and stability will be forthcoming. Such information will facilitate more definite evaluation of the adequacy of a particular ration in terms of pteroylglutamic acid.

#### SUMMARY

1. The effect of feeding diets low and high in pteroylglutamic acid on the performance of adult chickens and turkeys and turkey poults was determined.

2. When a basal diet containing 0.42 mg of PGA/kg of ration was fed to turkey or chicken hens no detrimental effect on egg production, hatchability, hemoglobin level or general appearance was demonstrated as compared to when 2.0 mg of PGA were added/kg of ration.

3. The amount of PGA found in the eggs of both chickens and turkeys was markedly lower when the basal diet was fed as compared to the amount found when the diet supplemented with PGA was fed.

4. The "apparent" free folic acid content of the blood was also lower for the turkeys fed the low PGA diet while the level in the blood of the chickens was relatively insensitive to differences in the dietary treatment. The amount of PGA observed after enzymatic digestion of the blood samples, however, was shown to be approximately the same for both groups of chickens and turkeys, regardless of dietary treatment.

5. A higher mortality and slower rate of growth were noted when both young poults and the hens were fed diets low in pteroylglutamic acid than when a supplemented diet was fed either to the hens, to the poults, or to both.

6. For poults from hens adequately supplemented with PGA, 0.8 mg of the vitamin/kg appeared to be adequate; however, for poults from hens fed the diet not supplemented



with PGA, the requirement is apparently greater than 0.8/kg of ration.

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# THE RELATION OF FED AND INJECTED TOCOPHEROLS TO DEVELOPMENT OF RANCIDITY IN THE STORED MEAT AND UTILIZATION OF CAROTENE BY THE RABBIT <sup>1</sup>

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Work has been done to show that alpha-tocopherol, which prolongs the induction period of the fat, is stored in the tissues of the rat and turkey (Criddle and Morgan, '47; Hanson et al., '44; Lundberg et al., '44; and Mason, '42). Watts, Cunha and Major ('46) found that the amount of tocopherol stored in hog fat was too small a quantity to be of practical value, especially when the animals were fed a natural ration.

In the work reported here, a study was made of the relation of the tocopherols to the prevention of development of rancidity in the stored meat and fat of the rabbit. In addition, the effect of tocopherols upon the utilization of carotene was investigated.

A number of authors (Davies and Moore, '41; Hickman et al., '42, '44a, '44b; Moore, '40; and Jensen, '46) have reported a sparing action of tocopherol upon vitamin A. Guggenheim ('44), Hove ('43, '44), Jensen et al. ('43), Quackenbush et al.

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('41, '42), and Sherman ('42) have shown also that the tocopherols are effective in enhancing the utilization of carotene. Harris et al. ('44) found that 0.5 mg of natural mixed tocopherols was the optimum daily dose to demonstrate the sparing action of carotene on rats.

## METHODS

### *Selection and care of animals*

American white rabbits weighing 500–700 gm were used for this experiment. They were weaned when 4 to 6 weeks old and fed commercial rabbit pellets until all were eating, as evidenced by maintenance of weight. They were then separated according to sex and put into 3 comparable groups, and placed on the experimental diet. Supplements to the diet were withheld until the vitamin A stores were depleted, as evidenced by leveling off of the growth curve.

### *Diet and supplements*

The composition of the purified diet is shown in table 1. Contrary to the findings of Mackenzie, Mackenzie and McCollum ('41), preliminary work with rabbits in our laboratory showed that fat was essential in the diet, both for utilization of carotene and general health and growth. For this reason lard was included in the diet.

Sufficient diet for 1 week was prepared at one time by mixing thoroughly all of the ingredients of the diet and then moistening the mixture sufficiently to form a pellet. The slightly moist pellets were spread on screens at room temperature overnight to dry and stored in closed tin cans for the week. The peroxide values of the diet at the beginning and end of the week were followed until it was apparent that there was no appreciable increase during this period. At first the yeast was ether-extracted, but this practice was soon discontinued because of the extremely low tocopherol content (Veron, '46). The diet was fed *ad libitum* in metal containers.

Supplements containing carotene, viosterol, and mixed tocopherols were prepared for each group weekly. Crystalline carotene (90% beta and 10% alpha) was dissolved in chloroform in 1-gram lots; the solution was taken up in ethyl laurate; and the chloroform was then evaporated on a water bath. The volume of the carotene solution was adjusted with ethyl laurate so that 1 ml of the solution contained 20 mg carotene. It was then stored in brown bottles in the refrigerator. Aliquots of this solution were used in mixing the weekly supplement. The vitamin D used was viosterol in oil with

TABLE 1  
*Composition of rabbit diet.*

BASAL DIET		SUPPLEMENTS		
		Tocopherol		
		low	high	
	%	mg/day	mg/day	
Casein	15	Tocopherol	1.0	11.1
Brewers' Yeast	15	Viosterol	1.0	1.0
Lard	10	Carotene	3.0	3.0
Wheat Bran	15			
Salt Mix <sup>1</sup>	6			
Cornstarch	39			

<sup>1</sup> Salt mix no. 51 (Mackenzie, Mackenzie and McCollum, '39).

standardized potency of 10,000 U.S.P. units per gm. A tocopherol concentrate containing 34% mixed tocopherols was used.

For the groups fed high levels both the viosterol and tocopherol were weighed directly into brown bottles, the carotene solution added, and the volume adjusted with ethyl laurate so that 0.3 ml was fed at each supplementation. The mixed supplement was then stored in the refrigerator. This supplement was fed by pipette directly into the animal's mouth 3 times weekly.

For the groups of animals receiving injected tocopherol, the viosterol and carotene were prepared and fed according to the method followed for the previous groups. The tocopherol, however, for this group was weighed out and the volume adjusted with ethyl laurate so that 0.5 ml contained the weekly

supplement which was injected with hypodermic syringe into the heaviest portion of the right thigh once weekly. All injections were kept within a 1-inch circle on the thigh.

The daily tocopherol requirement of the rabbit has been found to be between 0.2 mg and 1.0 mg per kilogram body weight (Eppstein and Morgulis, '40, '42; Friedman and Mat-till, '41; Hove and Harris, '47; Mackenzie, Levine and McCollum, '40; Mackenzie and McCollum, '39, '40). The amount of 1.0 mg mixed tocopherol was chosen for the low level group to avoid any incidence of dystrophy.

### *Treatment of the meat*

At the end of the experimental period, the rabbits were dressed, the livers and free fat removed and frozen, and the meat cut from the bones and frozen. The right thigh of the animals which had had the tocopherol injected was removed and stored separately. After freezing, the meat of each animal was ground and the sausage mixed thoroughly. It was found much easier to get a fine grind, free from stringy tissue, if it was ground while still frozen. The sausage was weighed out into 50 gm lots, which were made into balls of similar size and shape and placed in freezer cartons with strips of wax paper between them to facilitate removal in individual balls for analysis. The cartons were then put into frozen storage at  $-18^{\circ}\text{C}$ .

### *Rancidity tests on the frozen sausage*

The samples were analyzed at intervals until the sausage from each animal was found rancid. The fat was extracted from the sausage by blending 50 gm sausage in a Waring blender for 1 minute with 100 mg chloroform and 70 gm of anhydrous sodium sulfate. The extract was filtered through Whatman no. 2 filter paper, and a 5 ml sample of the clear filtrate dried to determine fat content. The amount of the filtrate necessary to make 0.5 gm fat was used for the test. A peroxide value above 10 was designated rancid. The

method used for determination of peroxide values throughout the experiment was reported by Watts and Major ('46).

### *Vitamin A and carotene analyses*

Vitamin A analyses were made on all livers using the Carr-Price reaction as reported by Davies ('33). This was changed to the Gallup and Hoefer method ('46) when good agreement was found between the two. Carotene was determined by the Wall and Kelley method ('43).

### *Determination of pH*

Five grams of sausage were mixed thoroughly with 50 ml distilled water. After 30 minutes the pH was read on a Beckman meter.

## EXPERIMENTAL RESULTS AND DISCUSSION

This work was carried out in 2 separate experiments. Experiment 1 included 3 groups of animals fed the purified diet. Group 1 received a low-level tocopherol supplement; group 2, a high-level tocopherol supplement; and group 3, a high-level tocopherol supplement by injection. Experiment 2 was concerned with animals fed a natural diet which was supplemented with injected tocopherol and tocopherol phosphate.

### *Experiment 1*

*Part A.* This experiment was set up using the purified diet shown in table 1. In addition to the effect of fed and injected tocopherol on development of rancidity, the effect of fed tocopherol on the utilization of carotene was estimated.

The effect of tocopherol upon rancidity development in the stored meat is summarized in table 2. At the end of 12 months' storage, all but one of the samples from the group fed low level tocopherol were rancid (mean peroxide value of 14), whereas none of those from the group fed high level tocopherol were rancid (mean peroxide value of 4.4). The mean peroxide value of the group which had the tocopherol injected was

between the average of these 2 groups, or 5.6. A statistical analysis of these figures shows that the difference between the average peroxide values of the high level of tocopherol, whether fed or injected, and the low level of tocopherol is significant at the 1% level.

TABLE 2  
*Effect of feeding and injecting tocopherol on the rancidity development of the meat.*

CODE NUMBER	TOCOPHEROL	PEROXIDE VALUES AFTER STORAGE FOR		pH OF MEAT
		12 mo.	18 mo.	
	<i>mg/day</i>			
32	1.0	18.0		5.50
39	1.0	8.0	13.5	5.70
45	1.0	16.0		5.70
47	1.0	14.0		5.69
		Mean 14.0		
33	11.1	7.2	14.0	6.15
34	11.1	4.0	9.0	5.55
37	11.1	3.4	5.0	5.90
40	11.1	3.0	5.0	5.50
	Injected	Mean 4.4 <sup>1</sup>	8.2	
35	11.1	3.0	6.5	6.00
36	11.1	7.8	9.0	6.04
38	11.1	4.0	6.5	5.85
42	11.1	5.0	5.5	5.70
43	11.1	7.0	8.5	5.70
44	11.1	7.0	9.0	5.62
		Mean 5.6 <sup>1</sup>	7.5	

<sup>1</sup> Significant at the 1% level. The method for determining significance was taken from Snedecor, G. W., *Statistical Methods*.

It has been reported that tocopherol in the diet is necessary to prevent the destruction of vitamin A and carotene in the digestive tract. More vitamin A was stored in the liver as well as more carotene excreted in the feces when the dietary tocopherol was increased (Guggenheim, '44; Harris et al., '44; Hickman et al., '42; Hove, '43). A carotene balance was run to see if the same conditions were necessary for carotene utilization for rabbits as had been reported for rats.

Before the carotene balance was started, the animals had been maintained on the same supplements for a period of 30

days or more. A 4-day period was used as a test period, and feces were collected at a stated hour daily. Feces that could not be analyzed for carotene content immediately after collection were frozen until an analysis could be made.

The results of the vitamin A storage and carotene excretion from this experiment are shown in table 3. The lower figure of carotene excreted by the group of animals fed a low level of tocopherol appeared to be due to an extremely small amount of feces on the first day of collection (4 gm as compared to a daily average of 35 gm), rather than to any difference in

TABLE 3  
*Effect of tocopherol on excretion of carotene  
and storage of vitamin A.*

PART	GROUP	NO. OF ANIMALS	SUPPLEMENTS FED		CAROTENE EXCRETED (AVERAGE)	AVERAGE VITAMIN A STORED IN LIVER	
			Tocopherol	Carotene		mg/gm	total mg
			mg/day	mg/day	mg/animal/day		
A	low	4	1.0	3.0	0.943	0.020	2.116
	high	4	11.1	3.0	1.597	0.018	1.570
	injected	6	11.1	3.0	1.327	0.014	1.298
B	low	6	1.0	1.5	0.688	0.012	1.231
	high	7	11.1	1.5	0.414	0.010	1.234

carotene utilization. The carotene content of the feces from this group on the day mentioned was even lower per gram than that on other days.

There was no significant difference between the 3 groups in either carotene excretion or vitamin A storage in the liver.

*Part B.* Since the results of the carotene balance and of the vitamin A assay on the livers (shown in table 3, part A) did not agree with those reported by Guggenheim on rats ('44), it was thought that another set of animals should be used to investigate the effect of the amount of tocopherol fed on carotene excretion, utilization, and rancidity development in meat. Because of the high quantity of carotene excreted and vitamin A stored in the liver, it was believed that the amount fed in part A was too far in excess of the requirement



to be a good measure of the effect of tocopherol (Moore, '40). Therefore, for this trial, the carotene level was decreased from 3.0 mg daily to 1.5 mg daily. Because Fraps and Meinke ('45) and Callison and Orent-Keiles ('46) had reported that the availability of carotene was greater when fed in an oil, the supplements were mixed and fed in peanut oil<sup>4</sup> instead of in ethyl laurate.

The results on carotene excretion and vitamin A storage for both parts of this experiment are shown in table 3. In neither experiment did the greater amount of tocopherol fed result in significant change in amount of carotene excreted nor in the amount of vitamin A stored in the liver. The tocopherol supplement, even in the low level, may have been sufficient to exert protective action on the carotene. More work is needed before conclusions can be drawn on the effect of feeding the supplements in oil instead of in ethyl laurate. However, some fat, either in the diet or in the supplements, appeared necessary in order to get any storage of vitamin A in the liver. In preliminary work where both were omitted, no vitamin A was found in any of the livers, although 3.0 mg carotene was fed daily. This was found to be in agreement with other work (Callison and Orent-Keiles, '46; Hove and Harris, '46; Quackenbush et al., '41, '42).

An accelerated method for development of rancidity in the ground meat was devised and compared with frozen storage results. This method consisted of mixing thoroughly 4.0 ml of chloroform with 25.0 gm of the ground meat ready for freezing. The mixture was put into 250 ml beakers in a layer of uniform thickness, the beaker capped with aluminum foil to prevent vaporization of the chloroform, and stored in an incubator at 30°C. In this way these samples could be stored for periods as long as 60 hours without apparent bacterial spoilage. Peroxide values were run on samples stored in this way at 36- and 48-hour intervals.

The results of the accelerated tests on the meat are shown in table 4. At the end of the 36-hour period all but one of

<sup>4</sup> Obtained through the courtesy of Planters Edible Oil Company, Suffolk, Va.

the meat samples from animals fed the low tocopherol level were rancid and that one was near the point of rancidity (average 17.1). On the other hand, only one of the samples from the animals fed high tocopherol showed any development of rancidity at the end of the 36-hour period (average 2.8). Statistical analysis shows that this difference is significant at the 1% level. The early development of rancidity in the one

TABLE 4

*Comparison of accelerated and frozen storage tests on sausage.*

CODE NUMBER	TOCOPHEROL FED	PEROXIDE VALUES			pH OF MEAT
		Accelerated	Frozen storage		
	<i>mg/day</i>	<i>36 hr.</i>	<i>48 hr.</i>	<i>6 mo.</i>	
63	1.0	13.7	19.5	11.5	5.83
66	1.0	23.2	37.0	11.5	5.80
69	1.0	26.5	41.5	21.0	5.72
70	1.0	20.0	35.0	14.5	5.60
72	1.0	8.5	22.0	8.5	5.65
74	1.0	10.5	23.5	9.5	5.73
		Mean 17.1 <sup>1</sup>	29.6	12.7	
61	11.1	12.5	25.0	11.0	5.73
62	11.1	0.1	1.5	1.0	5.70
64	11.1	3.5	4.3	0.1	5.72
65	11.1	0.5	0.7	3.5	5.83
68	11.1	0.0	0.5	3.5	5.70
71	11.1	3.0	4.0	4.5	5.70
73	11.1	0.0	3.7	5.0	5.68
		Mean 2.8 <sup>1</sup>	5.7	4.1	

<sup>1</sup> Significant at the 1% level.

sample cannot be explained on the basis of pH difference (Watts and Peng, '47a). A colorimetric comparison of filtrates of the meat (prepared by blending 100 gm sausage with 140 ml water) from the different animals showed no higher hemoglobin content in this sample (Watts and Peng, '47b).

Peroxide values were determined on the meat after 6 months in frozen storage. The relative values, as shown in table 4, agreed with those made earlier by accelerated tests.

*Experiment 2*

This experiment was set up to determine the effect of injected tocopherol and tocopherol phosphate on rancidity development in the stored meat of rabbits fed a natural diet.

The diet used was a commercial rabbit chow<sup>5</sup> with the following guaranteed percentage composition: crude protein, not less than 14.5%; crude fat, not less than 2.0%; crude fiber, not more than 18.0%; nitrogen free extract, not less than 44% and ash, not more than 7.5%. The ingredients were ground oats, corn meal, soybean oil meal, corn germ meal, alfalfa meal, wheat gray middlings, molasses, riboflavin supplement, 1.5% calcium carbonate, and 0.5% iodized salt.

The animals selected for this experiment were 3 to 4 months old and had nearly reached full growth. They were divided into 2 lots; each lot consisted of a control group receiving no supplements and an experimental group. Lot 1 experimental group was injected with 200 mg of mixed tocopherol in ethyl laurate at the beginning and mid-point of a 3-week period. For lot 2 experimental group, 200 mg of water-soluble tocopherol phosphate was injected at these same intervals.

At the end of the 3-week period the animals were dressed and the right thigh of the injected group separated before storage, as has been described under the method.

The results of the experiment are shown in table 5. In the frozen storage tests of the ground meat no difference was shown between the animals injected with tocopherol or tocopherol phosphate and those of the control groups receiving no supplementary tocopherol. However, when compared to the rest of the tissue, protection from rancidity development was shown, in both lots, in the thigh muscle at the site of injection of the tocopherol.

It may be that the time between the injection of the tocopherol and killing of the animals was too short to allow for storage throughout the tissues, although Eppstein and Morgulis ('42) found that a 3-10-day period was sufficient

<sup>5</sup> Distributed by the Ralston Purina Company.

to cure rabbits of advanced dystrophy when they administered the water soluble disodium- $\alpha$ -tocopherol phosphate by injection. This experiment allowed fully the 10-day period suggested by Eppstein and Morgulis. Hove and Harris ('47) also found that injected tocopherol-phosphate brought about the cure of muscular dystrophy in the rabbit in a 5-25-day period.

TABLE 5

*Effect of injecting tocopherol and tocopherol-phosphate.  
(Mature animals on natural diet.)*

CODE NUMBER	AMT. TOCOPHEROL INJECTED (MG)	STORED MEAT PEROXIDE VALUES			PH OF MEAT
	Mixed tocopherols				
<i>Lot 1</i>		<i>5 mo.</i>	<i>7 mo.</i>	<i>9 mo.</i>	
21	0.0		16.0		6.60
22	0.0		12.0		6.20
23	0.0		8.0		6.17
24	400.0		7.0		6.10
25	400.0		17.0		6.11
25 <sup>1</sup>				11.0	
26	400.0		5.0		6.03
<i>Lot 2</i>	Tocopherol phosphate				
50	0.0	2.8			6.14
51	0.0	2.8			5.90
52	0.0	15.7			5.91
53	400.0	72.7			5.96
54	400.0	20.0			5.80
54 <sup>1</sup>				7.5	
55	400.0	29.5			5.78
55 <sup>1</sup>				9.5	
56	400.0	7.2			6.02
56 <sup>1</sup>				4.5	

<sup>1</sup> Muscle at site of injection.

#### SUMMARY AND CONCLUSIONS

The relation of tocopherols in the diet to development of rancidity in the fat and meat and to the utilization of carotene was investigated.

1. When high levels of tocopherol were fed to or injected in rabbits on a purified diet, protection from the development of rancidity was shown.

2. On a natural ration, no protection from the development of rancidity was shown when the animals were injected with tocopherol or tocopherol phosphate 3 weeks before slaughter.

3. Accelerated tests for the development of rancidity on sausage preserved with chloroform agreed with tests made on sausage held in frozen storage.

4. No difference either in the utilization of carotene or in the liver storage of vitamin A was observed on supplementing a synthetic diet with high and low levels of tocopherol.

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# THE EFFECT OF DIETARY RESTRICTION OF B-COMPLEX VITAMINS AND PROTEIN ON THE EXCRETION OF CREATININE BY HUMAN SUBJECTS <sup>1,2</sup>

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## FOUR FIGURES

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The work of Folin ('05), Hoogenhuyze and Verploegh ('05), Klercker ('07), and Shaffer ('08) some 40 years ago led to the formulation of several concepts which, in spite of intensive investigation and much individual disagreements, still are of interest to the nutritionist: (1) that some biochemical phenomena proceed at similar, apparently constant,

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rates in all healthy physically fit individuals, quite independent of the usual variations of the normal diet and physical activities and, therefore, may be used as "yard sticks" for assessing the total active mass of certain types of tissues; and (2) that alterations of structure or functional efficiency of such tissues resulting from disease, malnutrition or other debilitating conditions may be reflected in altered rates of the basic reactions.

Although the first is opposed to modern concepts of extreme complexity and variability of biochemical phenomena, nevertheless, the early investigators demonstrated such an apparent constancy in the case of creatinine in relation to the unit weight — the creatinine coefficient — which presumably is related to the muscular mass (Shaffer, '08; Hodgson and Lewis, '26; McClugage, Booth and Evans, '31). This was all the more surprising since the conclusions were based upon determinations of the daily rate of urinary excretion which, in addition to cellular mechanisms, involves renal function. In recent years the role of protein and amino acids in the synthesis of creatine and creatinine has received particular attention. The result has been an increasing emphasis on the variation rather than on the uniformity of the creatinine excretion. The changing point of view is evident in the reviews of Hunter ('27), Peters and Van Slyke ('46) and Beard ('41).

The differences in the average daily rate of excretion were explained by Folin and Shaffer largely on the basis of the second postulate. This was perhaps best stated by Shaffer ('08) that "in a muscle in a high state of nutrition and development, certain processes which cannot be fully defined at present, but which lead to the formation of creatinine as a waste product, are proceeding at a greater speed than in a muscle organically weak or diseased" and that upon the intensity (see Hoogenhuyze and Verploegh, '05) of these processes, as measured by the creatinine coefficient, "appears to depend the muscular efficiency of the individual." Because of the experimental difficulties and the long time required in

such investigations, these implied relations have not been studied in a satisfactory manner in human subjects.

In the course of an experiment with 7 initially physically fit young men (Berryman et al., '47), an opportunity was presented for applying uniform procedures to a study of the variations of creatinine excretion and their relation to changes in weight and physical performance over a period of 50 weeks as follows: a "normal" period of 11 weeks; an experimental period of 8 months (36 weeks) during 4 of which the diet was restricted with respect to protein and B-complex vitamins, followed by 4 months in which a program of supplementation was instituted in accordance with individual needs; and a final period of 3 weeks, during which all subjects received approximately equal quantities of a rehabilitation diet. Two of the subjects served as "controls" on the effect of the restricted basal diet plus all of the supplements during the 2 periods of 4 months each. Thus, the experiment provided data on the "constancy" of the creatinine coefficient under controlled conditions and the effect of subsequent changes in the "state of nutrition."

#### EXPERIMENTAL

Since the experimental conditions were described fully by Berryman et al. ('47) in connection with other data, only the salient features will be given here. The 7 men, ages 22 to 27 years, were chosen from many volunteers on the basis of physical and mental qualities. All subjects received equal weighed quantities of "normal" diet I during the first 11 weeks, during which a uniform program of training and test procedures was instituted and an apparent plateau of physical performance was attained. The diet contained (Berryman et al., '47): 3170 cal.; 70 gm protein (including protein from 75 gm lean beef, 225 ml milk and 1 egg); 1.44 mg thiamine; 1.84 mg riboflavin; 15.6 mg niacin; 44  $\mu$ g biotin; 64  $\mu$ g L. casei factor; 4.7 mg pantothenic acid; 1.7 mg pyridoxine; and adequate quantities of ascorbic acid, vitamin A, calcium, phosphorus, and iron.

The basal weighed experimental diet II was then given to all subjects: a total of only 5 weeks (12 to 16, inclusive) to subjects 3 and 4, and 36 weeks (12 to 47, inclusive) to the other 5 subjects. This permitted a determination of the initial creatinine excretion on a relatively creatine and creatinine-free diet by the 5 experimental subjects and *subjects 3 and 4*

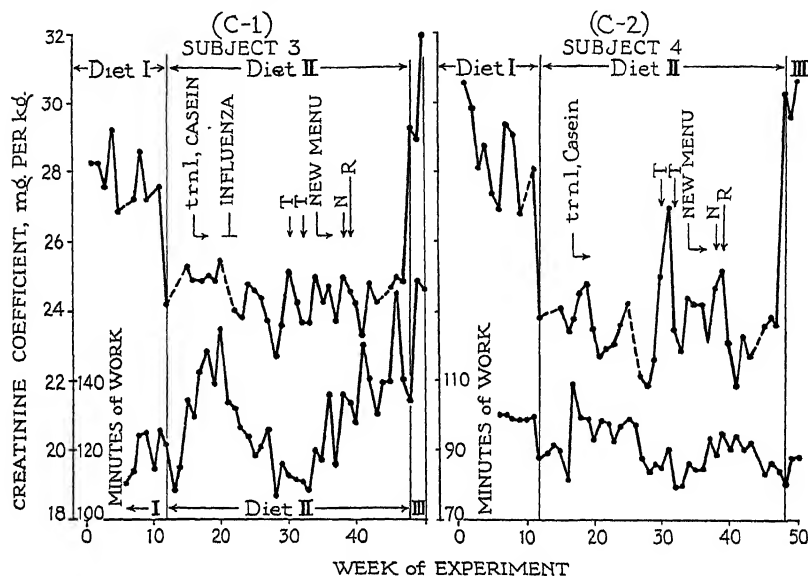


Fig. 1 Control subjects. Creatinine coefficient and work output expressed in total minutes of work to exhaustion in 2 work periods. The letters refer to vitamin supplementation—small letters to vitamins given orally in tablets 3 times per day in quantities sufficient to bring the total intake to the level of diet I; capital letters to vitamins given intravenously in large doses—as follows: t or T, thiamine; r or R, riboflavin; n or N nicotinamide; l, “lesser known” B-complex vitamins, pyridoxine, pantothenic acid, biotin, and L. casei factor.

*who subsequently served as “controls” on the effect of the basal diet plus supplements.* The diet contained: 3300 cal.; 45 gm protein (including animal protein only from salt pork without visible lean meat); 0.50 mg thiamine; 0.30 mg riboflavin; 5.8 mg niacin; 19  $\mu$ g biotin; 23  $\mu$ g L. casei factor; 1.1 mg pyridoxine; 1.1 mg pantothenic acid; and adequate quantities of ascorbic acid, vitamins A and D, and minerals either in the diet or in supplements.

Supplements were then given to the control subjects 3 and 4: 40 gm of protein in the form of calcium caseinate and the 7 crystalline vitamins indicated in both diets above, in quantities equal to that of diet I. These subjects later received

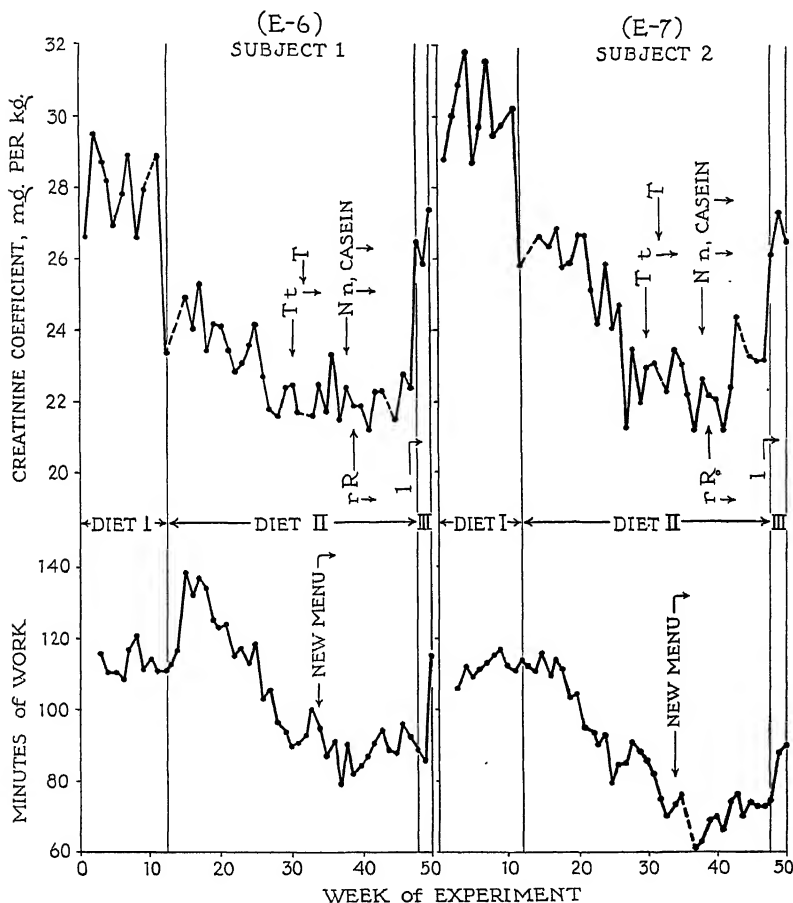


Fig. 2 Experimental subjects 1 and 2. See figure 1.

intravenous injections of vitamins at the same time as the experimental subjects (see capital letters in figures 1 to 4).

The month in which protein supplementation was instituted in the 5 experimental subjects is shown in tables 1 and 2; the

order of vitamin and other supplementation is indicated in figures 1 to 4. This had to be changed somewhat in individual cases because of the symptoms which developed in some subjects. Thus protein (casein) supplementation was instituted as follows: at the beginning of the fifth month to experimental subjects 5, 6, and 7, and at the beginning of the seventh month to experimental subjects 1 and 2. In addition, subjects 6 and 7 received further supplements of 100 gm lean

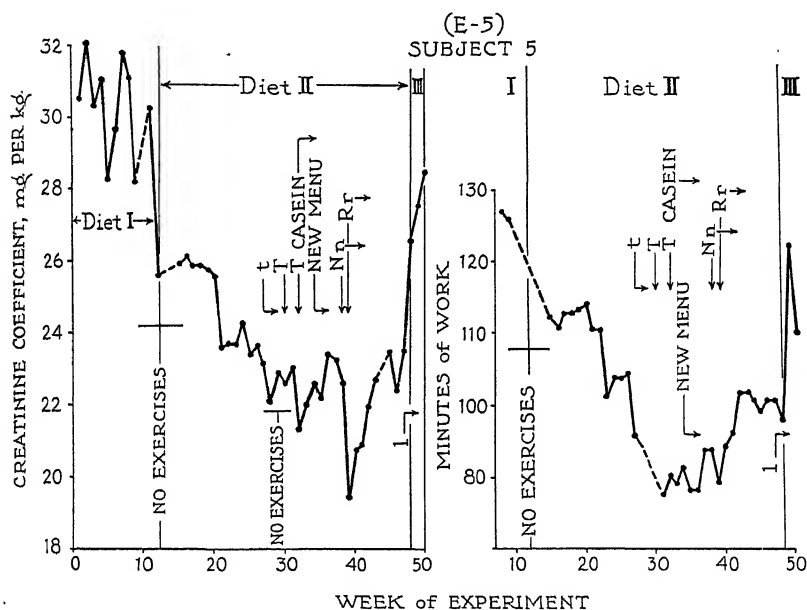


Fig. 3 Experimental subject 5. See figure 1.

beef, 225 ml milk and 1 egg at the beginning of the seventh month.

Rehabilitation diet III was given during weeks 48 to 50, inclusive. It contained 250 to 350 gm meat, 1 to 1.5 liters milk, 1 to 2 eggs, dried brewers' yeast, and, in addition, all of the crystalline B-complex vitamins. All subjects were served the same generous portions and, although they were allowed to ask for additional food, they seldom ate more. Thus, the in-

take of food, although not weighed as in the previous diets, was approximately the same for all subjects.

*Weight.* This was determined each morning after urine had been voided at 7 A.M. and before any water had been taken.

*Work output.* This was determined with a calibrated electro-dynamic brake bicycle (Kelso and Hellebrandt, '34). Work was performed twice to the point of exhaustion of the leg

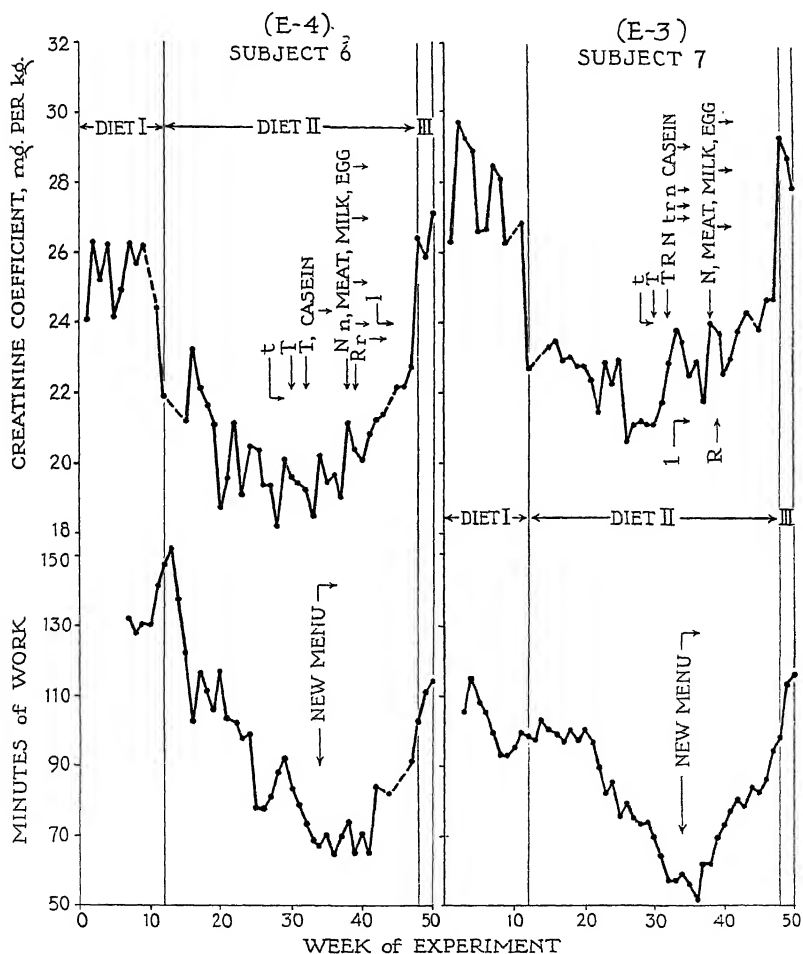


Fig. 4 Experimental subjects 6 and 7. See figure 1.

muscles, with a rest period of exactly 10 minutes between the tests (Foltz, Ivy and Barborka, '42). Previous experience had shown that the rate of pedaling is very important in obtaining comparative results from many subjects. Accordingly, throughout the year all subjects pedaled at a rate of  $92 \pm 4$

TABLE 1

*Effect of normal and rehabilitation diets. Monthly averages.*

SUB- JECT NO.	NORMAL DIET I			REHABIL. DIET III	DIFFERENCES BETWEEN RESULTS OF I AND III	
	July Weeks 1-2	Aug. Weeks 3-7	Sept. Weeks 8-11	June Weeks 48-50	Sept. and June	
Creatinine, gm per day						
3	2.27	2.15	2.13	2.41	mg + 280	% + 13.1
4	2.04	1.87	1.84	2.02	+ 180	+ 9.8
1	1.76	1.77	1.73	1.69	— 40	— 2.3
2	2.16	2.21	2.14	1.88	— 260	— 12.1
5	2.21	2.08	2.04	1.89	— 150	— 7.4
6	2.16	2.13	2.12	2.18	+ 60	+ 2.8
7	1.96	1.89	1.80	1.93	+ 130	+ 7.2
Creatinine coefficient, mg per kg per day						
3	28.3	27.6	27.9	30.0		
4	30.3	28.1	28.0	30.2		
1	28.1	28.1	27.8	26.6		
2	29.4	30.5	29.8	26.7		
5	31.3	30.2	29.8	27.5		
6	25.2	25.4	25.5	26.5		
7	28.2	28.0	27.1	28.6		
Weight, kg						
					kg	%
3	80.1	77.9	76.8	80.2	+ 3.4	+ 4.4
4	67.4	66.5	65.8	66.9	+ 1.1	+ 1.7
1	62.8	62.5	62.3	63.6	+ 1.3	+ 2.1
2	73.5	72.3	71.7	70.3	— 1.4	— 2.0
5	70.5	68.8	68.3	68.8	+ 0.5	+ 0.7
6	85.7	84.0	83.2	82.2	— 1.0	— 1.2
7	69.8	67.6	66.6	67.3	+ 0.7	+ 1.1

TABLE 2

*Effect of restricted diet and supplements. Monthly averages.*

The initiation of protein supplementation is indicated by *c* (45 gm casein) and *m* (100 gm lean beef, 225 ml milk, 1 egg). The numbers in parentheses in the next to last column indicate the month in which the minimum was attained; for example, (1) and (2) represent January and February, respectively.

SUB- JECT NO.	OCT. Weeks 12-16	NOV. Weeks 17-20	DEC. Weeks 21-24	JAN. Weeks 25-29	FEB. Weeks 30-33	MARCH Weeks 34-37	APRIL Weeks 38-41	MAY Weeks 42-46	Jan.	CHANGE		
										Minimum		May
Creatinine, gm per day										% of initial (Oct.=100%)		
3	1.90	1.91 <i>c</i>	1.83	1.81	1.82	1.85	1.86	1.91	— 4.7	— 4.1(1)	+	0.5
4	1.56	1.57 <i>c</i>	1.49	1.46	1.58	1.54	1.52	1.51	— 6.4	— 6.4(1)	—	3.8
1	1.51	1.51	1.44	1.40	1.35	1.38	1.37 <i>c</i>	1.42	— 7.3	—10.6(2)	—	6.0
2	1.88	1.84	1.74	1.58	1.54	1.48	1.47 <i>c</i>	1.59	—16.0	—21.8(4)	—	15.4
5	1.78	1.75	1.59	1.51 <i>c</i>	1.42 <i>c</i>	1.47	1.37	1.51	—15.2	—23.0(4)	—	15.2
6	1.84	1.71	1.63	1.54	1.47 <i>c</i>	1.50	1.59 <i>m</i>	1.68	—16.3	—20.1(2)	—	8.7
7	1.54	1.50	1.44	1.39	1.41 <i>c</i>	1.37	1.44 <i>m</i>	1.56	— 9.7	—11.0(3)	+	1.3
Creatinine coefficient, mg per kg per day												
3	24.8	25.1 <i>c</i>	24.2	23.8	24.2	24.5	24.3	24.7				
4	23.8	24.2 <i>c</i>	23.1	22.7	24.6	24.0	23.7	23.4				
1	24.1	24.3	23.3	22.5	21.9	22.3	21.9 <i>c</i>	22.2				
2	26.3	26.3	25.5	23.1	22.8	22.5	22.1 <i>c</i>	23.3				
5	25.9	25.8	23.8	23.0	22.3 <i>c</i>	22.9	20.9	22.7				
6	22.2	21.0	20.2	19.5	19.2 <i>c</i>	19.7	20.6 <i>m</i>	21.8				
7	23.2	22.9	22.2	21.4	22.4 <i>c</i>	22.7	23.3 <i>m</i>	24.2				
Weight, kg												
3	77.0	76.3 <i>c</i>	75.5	75.9	75.2	75.6	76.6	77.4	—1.4	—2.3(2)	+	0.5
4	65.9	64.9 <i>c</i>	64.8	64.5	64.4	64.1	64.3	64.6	—2.1	—2.7(3)	—	2.0
1	62.9	62.3	62.0	62.1	61.3	61.1	62.6 <i>c</i>	63.6	—1.3	—2.9(3)	+	1.1
2	71.7	69.9	68.3	68.3	67.5	65.7	66.6 <i>c</i>	68.3	—4.7	—8.4(3)	—	4.7
5	68.8	67.8	66.8	65.7	63.6 <i>c</i>	64.0	65.6	66.6	—4.5	—7.6(2)	—	3.2
6	83.1	81.6	80.8	78.7	76.5 <i>c</i>	76.1	77.0 <i>m</i>	77.3	—5.3	—8.4(3)	—	7.0
7	66.7	65.7	65.0	64.0	62.0 <i>c</i>	60.5	61.6 <i>m</i>	64.6	—4.0	—9.3(3)	—	3.8



revolutions per minute. In order to eliminate the complication of increasing performance due to training, all subjects performed the test 3 times each week, along with other similar tests during the first 2 months. Thereafter, the activities were decreased and the work output was determined only once each week. During the first month, the field strength of the generator, or the resistance against which the work was performed, was so adjusted that each subject became exhausted within 50 to 60 seconds in each of the 2 work periods. The rate of work finally adopted was 19.1 kgm per second for subjects 1 and 5, and 23.8 kgm per second for subjects 2, 3, 4, 6, and 7.

*Urine.* Collections were made 4 days each week in exactly 24-hour periods, from 7 A.M. Tuesday to 7 A.M. Saturday. The 1-gallon amber glass jugs were kept in refrigerators at all times. The volume was measured each morning, and exactly one-tenth volume was placed into a bottle containing the 4-day composite sample. The latter was kept in the deep-freeze unit.

*Creatinine.* The photoelectric method of Peters ('42) was used. Dilutions were made to give galvanometer deflections in the range of 40 to 60 units, using the Evelyn colorimeter. A difference of 1.0 unit, from 59 to 60, represents an error of 3.2%; from 40 to 41, an error of 2.8%. In 100 consecutive duplicate analyses, the mean analytical error was well within  $\pm 1\%$ . The procedure was standardized at intervals and checked by means of recoveries of added creatinine, using creatinine-zinc chloride and creatinine picrate. Throughout the experiment, all determinations were made by the same analyst.

## RESULTS

The most rapid changes in weight during the first period occurred in July and August while the subjects were undergoing training. However, the greatest losses were noted during the first week. This is not apparent in the average results given in table 1. Thereafter, the weight remained well within

$\pm 1$  kg during September and the succeeding 2 months of the restricted diet II (table 2).

The weight of 4 of the subjects (2, 5, 6, and 7) began to fall quite rapidly in December (the third month of the restricted diet) when early symptoms of deficiency began to appear. Diet II became monotonous, and at this time all of the subjects began to refuse some of the corn meal biscuits and the canned vegetables. The quantity of food thus left unconsumed was variable from day to day. It was small at first, but became larger during January, amounting to from 50 to as high as 800 cal. on some days, with an average of about 200 cal. The control subjects, particularly subject 3, refused as much food as the experimental subjects. They ate all of the food when a new attractive menu, which had the same ingredients as before, was given after March 1.

The work output, expressed in minutes required for exhaustion in 2 work periods, is shown in figures 1 to 4. The test is not entirely objective; it is subject to training, and periods of "staleness" and, like all physical efficiency tests, requires cooperation. However, it is improbable that any of these factors was of major importance because of the precautions taken against the occurrence of staleness and continued training response, and because of each subject's conscientious personal desire to do his best in each test. The parallel changes which were observed with other tests during the periods of restriction and supplementation were discussed in a previous paper (Berryman et al., '47).

#### *Excretion of creatinine and the creatinine coefficient*

*Weekly variations.* Since the variations of weight were small from week to week, the coefficient generally paralleled the total daily excretion. The results shown in figures 1 to 4 represent the average creatinine coefficients over periods of 4 days each week. Although the average tended to minimize the daily variations, considerable change was noted from week to week. The results are plotted on a large scale, which thus exaggerates the differences, and perhaps can be judged

best by the calculated coefficients from control subjects 3 and 4 during the first 2 months of diet II. Thus, the maximum deviations from the mean of all determinations during October and November were:  $-3.0$  and  $+2.2\%$  from subject 3;  $-2.5$  and  $+3.3\%$  from subject 4. In the case of subject 3, the maximum deviations remained within this range during the entire 8 months of diet II; however, they became greater in later months in the case of subject 4, the greatest differences of  $-6.2$  and  $+11.1\%$  of a 2-months' average occurring during February and March.

The maximum variations of the coefficients from the 2-months' mean of each subject were somewhat greater with diet I which contained creatinine. Thus, during August and September, the maximum percentage deviations, in the order of subjects given in the tables, were:  $-3.1$ ,  $+5.6$ ;  $-4.5$ ,  $+4.8$ ;  $-4.8$ ,  $+3.2$ ;  $-4.8$ ,  $+3.4$ ;  $-6.0$ ,  $+3.7$ ;  $-4.9$ ,  $+3.3$ ;  $-4.9$ ,  $+6.0$ .

Thus, the tendency toward variations was greater in some subjects than in others, which is evident not only in our data (figs. 1 to 4) but also in those of Folin ('05), Hoogenhuyze and Verploegh ('05), and Shaffer ('08).

In many instances, the excretion increased or declined in a regular manner over periods of several weeks during the administration of diet I. These phenomena did not occur simultaneously in other subjects. Other instances of regular decline or rise were noted during the long period over which the restricted diet II was administered.

*Monthly variations. Normal diet I and first month of diet II; 8 months of diet II in case of subjects 3 and 4.* Because of the weekly variations in creatinine excretion, which often appeared to be rhythmic, a certain degree of variation in the monthly averages was to be expected. The total excretion generally decreased during the 3 months of diet I (table 1).

All subjects excreted considerably less creatinine after initiation of the almost creatinine-free diet II. Thus, they excreted an average of 220 to 280 mg per day less in October than in the preceding month. In the case of the control sub-

jects 3 and 4, the creatinine excretion declined gradually until it reached a minimum in January, after which, on the same dietary regimen, it increased until, in May, it attained approximately the same level as in October (table 2).

These changes were not so apparent after calculation of the creatinine coefficient, which represents the quotient of the creatinine excretion expressed in milligrams, divided by the weight of the individual expressed in kilograms. Thus, if the excretion decreases or increases at the same percentage rate as the weight, then the coefficient remains unchanged. The coefficient reflects the relative changes in these 2 variables. During August and September, in the period of diet I, the coefficient increased slightly in the case of subjects 3 and 6, probably indicating that the weight decreased more rapidly than the excretion of creatinine. On the other hand, the coefficients decreased slightly in the case of the other 5 subjects; that is, the excretion decreased more rapidly than the weight. On the average, the 7 subjects excreted 2.16% less creatinine in September than in August, and the weight declined 1.13%. The differences are greater if the results from July and September are considered. During January, the fourth month of gradual decline, in the period of diet II, control subjects 3 and 4 excreted 4.7 and 6.4% less creatinine than in October, and the weight decreased only 1.4 and 2.1%, respectively (table 2).

On the whole, the downward trend of the creatinine excretion, although seemingly parallel, was at a greater rate than the loss of weight. Furthermore, the minima were not always attained during the same month. Thus, in the case of control subjects 3 and 4, the minimum weights were attained 1 and 2 months, respectively, after the minima of creatinine excretion (next to last column, table 2). In only 1 instance (subject 7), both minima occurred during the same month; however, here also the change with respect to creatinine excretion, — 11.0%, was greater than the weight, — 9.3%.

The rise in creatinine excretion which occurred from February to May often was accompanied by a rise in body weight.

However, the magnitude of the changes was not always equal (table 2). Although the changes with respect to weight and creatinine may have resulted from some fundamental alterations in metabolism, the effect, even though small, was not equal. In other words, the factors concerned with formation and excretion of creatinine appear to be subject to control and variation like all other biochemical phenomena. This point must be considered in any discussion concerning the creatinine coefficient.

However, in confirmation of the observations of Folin and Shaffer, it was found that the average daily excretion over a period of 1 month was to some extent related to the average weight of the individuals. Six of the subjects were muscularly well-developed, and had no evident excess fat. Subjects 2, 3, and 7 were tall and thin; subjects 4, 1, and 5 were stocky. Subject 6 was fat. The average daily creatinine excretion in September, in the order of decreasing weight (table 1, subjects 6, 3, 2, 5, 7, 4, 1) were: 2.12, 2.13, 2.14, 2.04, 1.80, 1.84, and 1.73 gm. The creatinine coefficients in the same order of subjects were: 25.5, 27.9, 29.8, 29.8, 27.1, 28.0, 27.8. On excluding the data from subject 6 who was fat, the highest coefficient was 4.9% above, and the lowest 4.6% below, the mean of 28.4 mg per kilogram per day.

The same approximate relation was observed in October, in the same order of subjects of decreasing weight as above, when diet II was given (table 2): 1.84, 1.90, 1.88, 1.78, 1.54, 1.56, 1.51 gm average creatinine excretion per day. The creatinine coefficients were: 22.2, 24.8, 26.3, 25.9, 23.2, 23.8, 24.1. Again, by excluding the first result, that of subject 6, the maximum deviations from the mean of 24.7 were — 6.1 and + 6.5%.

Since the subjects ate the same weighed diets and, therefore, metabolized about the same quantity of protein, the differences cannot be ascribed to differences of "intensity" of exogenous protein metabolism, as postulated by Beard ('41). This is further evident from the variable and negligible

effect of the addition of casein to the basal diet of subjects 3 and 4 in November (table 2).

*Effect of restricted diet II and supplementation.* From weeks 12 to 28, none of the experimental subjects received supplements of vitamins or protein in the basal diet. Like that of the control subjects, the creatinine excretion decreased, but to a greater degree. The excretion dropped rapidly in the case of subjects 2, 5, and 6. Clinical symptoms appeared in varying degrees in all subjects, and were evidenced in part by the following signs: decline in work output, as compared with that of the control subjects, which became most marked during weeks 21 to 24 (figs. 1 to 4); altered cardiovascular response to various work tests; scrotal skin lesions in subjects 1, 5, 6, and 7; irritability, insomnia, lack of power of concentration which forced 3 of the subjects to give up courses of study in the evening school; pain in the legs and ankles; and increased pyruvic acid content of the blood. In the case of subjects 2, 5, and 6, the marked decrease in creatinine excretion began with, and paralleled, the development of the deficiency symptoms. However, this was not so evident in the case of subjects 1 and 7 when the results were compared with similar changes in excretion of control subjects 3 and 4. The marked changes in creatinine excretion as related to weight in the control and experimental subjects are evident in the next to last column of table 2. The results suggest that the mechanisms concerned with the production and excretion of creatinine are relatively less affected in some subjects than in others.

The supplementation of vitamins was begun in February. Because of the variability of the weekly excretion and the long time required to obtain average results, the effect of individual supplements could not be studied. The welfare of the subjects had to be considered. The creatinine excretion of subjects 1 and 2 remained stationary during February and March when only thiamine had been given, and rose slightly during the succeeding months of April and May when other supplements were given. Subjects 5, 6, and 7 received

supplementations of casein along with the vitamins, beginning in February. Relatively little change in creatinine excretion was noted in this and the succeeding month. The physical condition of subjects 6 and 7 was not apparently improved and, therefore, it was decided to give additional supplements of 100 gm lean meat, 1 glass of milk, and 1 egg per day in April. Thus, the supplemented diet of these 2 subjects contained more protein than diet I; in fact, it contained 25 gm more of meat. As was to be expected, the creatinine excretion of subjects 6 and 7 rose, and in May was  $-8.7$  and  $+1.3\%$ , respectively, of the output of October (table 2). In spite of 2 months of complete supplementation, it was still below that of September (table 1). The work output of all of the experimental subjects, although improved, was still low in May after several months of complete supplementation.

The residual effects associated with dietary restrictions are further shown by a comparison of the results from September, diet I, and June, diet III (table 1). Subjects 3 and 4, who had received casein and vitamin supplements during 7 months of the preceding period of diet II, excreted 280 and 180 mg *more* of creatinine, or  $+13.1$  and  $+9.8\%$ , respectively, in June than in September. The previous diet, therefore, did not affect the response to diet III rich in meat and other dietary essentials.

On the other hand, subjects 1 and 2, who previously had received casein for 2 months and vitamins for 4 months, excreted 40 and 260 mg *less* ( $-2.3$  and  $-12.1\%$ ), respectively, than in September. Subject 5, who had previously received both supplements for 4 months, excreted 150 mg *less* ( $-7.4\%$ ), than in September. In contrast to this, subjects 6 and 7, who had received supplements similar to subject 5, but had also received meat, milk and eggs in the previous 2 months, excreted only 60 and 130 mg *more*, or  $+2.8$  and  $+7.2\%$ .

The weight of all subjects had returned to approximately the same level in June as in September. The differences in creatinine excretion, therefore, cannot be ascribed to dif-

ferences in weight. They cannot be explained entirely on the basis of the creatine or creatinine content of the diets, nor are they directly related to the protein content, which, as far as is known, is the most important source of nitrogen compounds for the synthesis of creatine and, presumably, also of creatinine. It is not likely that the differences were due to storage of creatine or creatinine because the period of 2 (subjects 1 and 2) to 4 (subjects 5, 6, and 7) months of protein supplementation before June was longer than the time usually considered necessary for nitrogen equilibrium.

Creatine determinations (by difference) were made toward the end of January, the fourth month of diet II, before supplementation was begun. The calculated excretions per day were as follows in the order of subjects 3, 4, 1, 2, 5, 6, and 7: 10, none, none, 80, 60, none, 20 mg.

#### DISCUSSION

The experimental conditions used in this study differed somewhat from those employed in previous studies on the factors concerned with the excretion of creatinine. All subjects received the same weighed quantities of food. The quantity of protein ingested, therefore, was the same in all subjects, regardless of weight. The restricted diet contained a considerable quantity of corn meal. The diets were administered over a long enough period to assure equilibrium and to permit observations on the variation in the excretion of creatinine. The subjects received an average of 3200 cal. per day, which was greater than had been given in most previous investigations. The diets were monotonous; each subject expected and looked for symptoms of deficiency; none of the subjects was aware of the experimental details or the results until the experiment was completed. At least once on each of 6 days each week, exercises were performed to the point of exhaustion which led to a high lactic acid content of the blood.

The factors of physical and emotional stress, which perhaps were greater in this long experiment than in previous experiments with laboratory personnel or students, should be con-



sidered in an evaluation of the results. These factors may have affected the nervous and hormonal mechanisms which exert some control not only on chemical reactions in the body tissues, but also on renal excretion. To some extent, they may have been responsible for the apparently periodic variations of the creatinine excretion which was observed in this experiment during the normal period as well as in the period of dietary restriction. However, these factors and others associated with loss of weight do not account for the gradually decreasing average rate of creatinine excretion which was observed during the period of dietary restriction, and which persisted for several months after supplementation of protein and B-complex vitamins.

Our results lend support to the theories of Folin and Shaffer mentioned in the introduction concerning the relation between the creatinine excretion and the physical and nutritional state of the individual.

#### SUMMARY AND CONCLUSIONS

— Seven men, ages 22 to 27 years, were subjected to a uniform regimen of physical exercises and tests over a period of 50 weeks, during which the variations of creatinine excretion, body weight, and physical performance were studied. The diets were as follows: a weighed, creatinine-containing normal diet I, containing 3170 cal., 70 gm protein and adequate quantities of minerals and vitamins, during 11 weeks; a weighed, essentially creatinine-free diet II, containing 3300 cal., 45 gm protein, greatly restricted quantities of B-complex vitamins, but otherwise adequate, over a period of 36 weeks — divided into equal periods of restriction and subsequent supplementations of thiamine, riboflavin, niacin, biotin, pteroylglutamic acid, pyridoxine, pantothenic acid and 40 gm casein; approximately equal quantities of a rehabilitation diet III, containing 250 to 350 gm of meat, during the last 3 weeks. After the fifth week of diet II, all supplements were given to 2 subjects who thus served as "controls" for a period of 7 months.

Creatinine was determined in composite samples collected over 4 days each week.

The creatinine excretion varied somewhat from week to week, often in a rhythmic manner, independent of the small changes of body weight in each subject. The maximum variations of the creatinine coefficient were of the order of  $\pm 5\%$  of each mean from all subjects during 9 weeks of diet I, and  $\pm 3\%$  of the mean from each of the control subjects during the first 9 weeks of diet II. These variations were minimized when the monthly averages were compared; the creatinine excretion then paralleled the rise or fall of body weight. During the first 5 weeks of diet II, the average coefficients of 6 subjects, weighing 62.0 to 77.0 kg, agreed within  $-1.5$  to  $+1.6$  mg, or  $-6.1$  to  $+6.5\%$  of the mean of 24.7 mg per kilogram per day; in the case of the seventh subject, who was fat and weighed 83.1 kg, the average coefficient was 22.2 mg. The average coefficients were in the same relative order of subjects with both diets I and II.

Administration of diet II to 5 subjects resulted in gradually decreasing average creatinine coefficients and physical performance which were paralleled to some extent by development of early clinical signs and symptoms of dietary deficiency. In 2 subjects, the administration of thiamine did not increase the average creatinine excretion or the coefficient within 2 months. Complete supplementation did not immediately increase the average creatinine excretion or the coefficient, nor did it rapidly improve the physical performance. In 2 subjects, the creatinine excretion and the coefficient were not raised to that of diet I when meat, milk and 1 egg were given in addition to all supplements (a total of about 110 gm protein) during the last 2 months of diet II. The effect of the previous dietary regimen was still evident during the period of the rehabilitation diet III, both by comparison of the results with those from the control subjects and by comparison of the individual data at the end of diet period II with those of diet period I.

Therefore, dietary restrictions with respect to protein and B-complex vitamins resulting in the development of deficiency symptoms may be reflected in metabolic changes associated with the formation and excretion of creatinine. These changes may persist, and are not rapidly abolished during subsequent supplementation.

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# THIAMINE DEFICIENCY IN THE CALF

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THREE FIGURES

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A synthetic milk diet which is satisfactory for the nutrition of the young calf has been reported (Wiese et al., '47a). Using this diet it has been found that nicotinic acid is not required by the newborn calf (Johnson et al., '47), while biotin and riboflavin deficiencies can be produced (Wiese et al., '46, '47b). In this paper we wish to report the results of the elimination of thiamine from the diet of the young dairy calf.

## EXPERIMENTAL

The composition of the thiamine-deficient "synthetic milk" ration fed is given in table 1. Ascorbic acid and nicotinic acid have been omitted, since previous work has demonstrated that they are not required (Wiese et al., '47a; Johnson et al., '47).

The experimental animals on this thiamine-free ration were male calves (5 Holsteins, 1 Jersey and 1 Red Poll-Jersey cross), cared for as previously reported (Wiese et al., '47a).

During the same experimental period, 7 other male calves (3 Holsteins, 3 Guernseys and 1 Jersey) were raised on the same diet with the addition of 0.65 mg of thiamine per kilogram of liquid ration and these animals served as simultaneous positive controls. The growth curves of the calves on this com-

plete ration are given in figure 1. In general, the growth rates of these controls are similar to those of positive controls previously reported from this laboratory (Johnson et al., '47), and quite similar to the Ragsdale ('44) normals. Jersey calf 33 twice suffered from respiratory infections — once during the first week, and again during the tenth and eleventh weeks of feeding. Both times the animal responded to sulfamerazine treatment, but its net gain was lower than normal, due to these 2 setbacks.

TABLE 1  
*Composition of synthetic milk.*

COMPONENTS	PER CENT	VITAMINS IN MG PER KG OF LIQUID "MILK," ADDED AT TIME OF FEEDING	
Casein (Labco)	30.0	Riboflavin	1.3
Lard	26.6	Pyridoxine	0.65
Salt mixture	4.0	Calcium pantothenate	2.6
Cerelose	39.4	$\alpha$ -Tocopherol acetate <sup>1</sup>	1.0
		2-Methyl-1,4-naphthoquinone	0.26
		Inositol	26.0
		Choline	260.0
		p-Aminobenzoic acid	2.6
		Pteroylglutamic acid	0.052
		Biotin	0.01
Vitamin A — 5,000 I.U. per day			
Vitamin D — 500 I.U. per day			

<sup>1</sup> The  $\alpha$ -tocopherol acetate and vitamin K dissolved in a small amount of lard, were homogenized along with the rest of the lard into the solution of casein, salts and cerelose.

The growth curves of the 7 calves on the thiamine-deficient ration are given in figure 2.

Two general types of symptoms were shown by the calves on the thiamine-low ration; one group (calves 27, 31, 32 and 39) showed acute symptoms, dying before a cure could be obtained; and the other group (calves 16, 17 and 36) showed a more chronic, and thus more strikingly characteristic, thiamine-deficiency syndrome. It is possible that calves 16 and 17 had better body stores of thiamine while calf 36 received

thiamine in its diet for the first 10 days. This may explain the fact that these animals were slower in coming down with the deficiency and showed more characteristic symptoms.

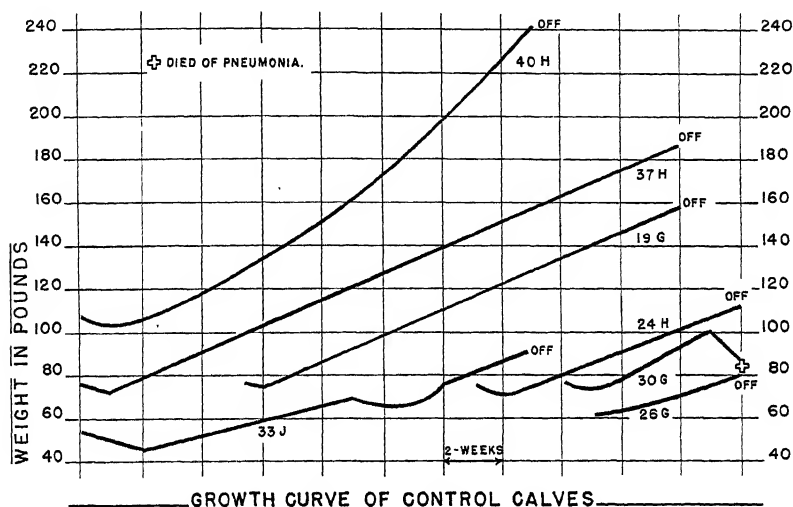


Fig. 1 Growth curves of control calves fed the complete thiamine-containing ration.

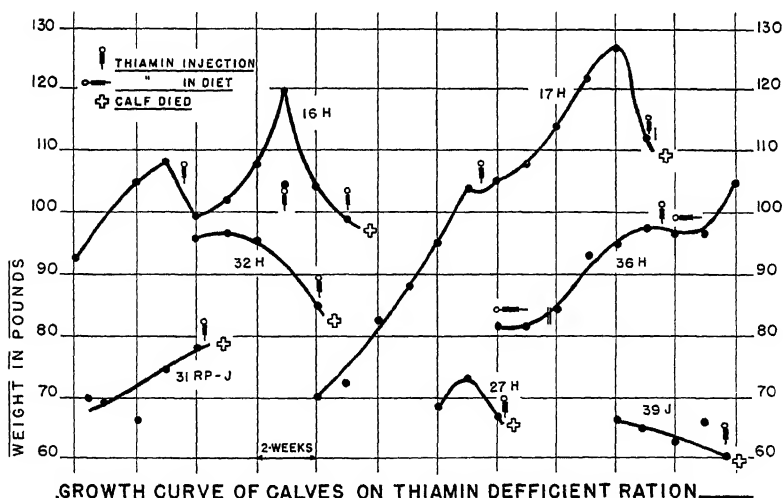


Fig. 2 Growth curves of calves fed the thiamine-low protein.

The acute syndrome consists of practically complete anorexia for 1 or 2 days, during which time there is very severe scouring, resulting within 24 hours in severe dehydration, collapsed veins and death. We have been unable to cure any of these severely dehydrated animals by intravenous injection of thiamine or thiamine plus 10% glucose solution.

Calves 16, 17 and 36, however, showed much more specific symptoms. These consisted of some anorexia, which appeared several days before any further symptoms, and was followed by the appearance of some mucous discharge from mouth and eyes, which was not as severe or prolonged as in the case of riboflavin deficiency. Later, poor coordination of the legs and marked weakness occurred. The animals were unable to stand, and when placed on their feet, their legs would spread out sideways, and the calf, having no control over them, would fall down. Two calves (17 and 36) had convulsions, while calf 16 showed constant muscle twitching and trembling. Some diarrhea occurred in these animals, but not as severe as in the first group, and there was little dehydration.

The response to the injection of 10 mg of thiamine in 10 ml normal saline, given intravenously, was almost miraculous in these 3 calves. The twitching and shaking stopped in 15 minutes, and within an hour calf 16 was able to eat. Calf 36, which had had 2 convulsions within one-half hour, was able to get up and stand normally within an hour after receiving the thiamine injection.

Calves 16 and 17 were given this 1 injection of 10 mg of thiamine at the time of their first severe symptoms, and then were not further treated until in the last stages of relapse which occurred 4-5 weeks later in both cases. At this time both calves showed the characteristic acute symptoms shown by calves 27, 31, 32 and 39, consisting of anorexia, scouring, severe weight loss, and dehydration. The injection of thiamine at this stage did not prevent the death of calves 16 and 17.

The fact that calves 16 and 17 responded to thiamine therapy and then later died during a relapse of symptoms which were

apparently the same as those shown by calves 27, 31, 32 and 39, is confirmatory evidence that the acute syndrome seen in these latter calves was in fact due to the absence of thiamine from the diet. This is further substantiated by the positive control animals, none of which showed these symptoms.

Calf 36 continued to receive thiamine in its diet after the initial injection, and this calf made an uneventful recovery.

Figure 3 pictures calf 17 when first deficient, showing the poor leg coordination and inability to stand alone.

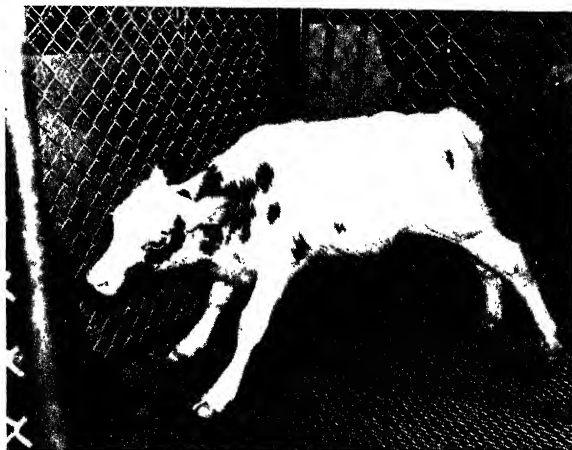


Fig. 3 Calf 17 — showing first symptoms of thiamine deficiency.

In general, 24-hour urine samples were collected from each calf once a week. These samples were analyzed for thiamine by the thiochrome procedure of Mickelsen, Cardiff and Keys ('45) and for pyruvate by the procedure of Li and Kato ('41). The urinary excretions of thiamine by the deficient calves and by some of the positive control calves are given in table 2.

Fasting post-absorptive blood samples were taken from the jugular vein each week and were analyzed for thiamine and for pyruvate. Urinary pyruvate excretion was also determined, and the blood and urine values obtained are given in table 3. In the case of the deficient calves, there are given wherever possible the last values obtained before symp-



TABLE 2  
*Urinary excretion of thiamine in  $\mu\text{g}$  per day.*

WEEKS ON DIET	APPROX. intake	REPRESENTATIVE POSITIVE CONTROL CALVES					CALVES ON THIAMINE-DEFICIENT RATION						
		24	26	30	33	40	16	17	31	32	36	39	
	mg/day	$\mu\text{g}$	$\mu\text{g}$	$\mu\text{g}$	$\mu\text{g}$	$\mu\text{g}$	$\mu\text{g}$	$\mu\text{g}$	$\mu\text{g}$	$\mu\text{g}$	$\mu\text{g}$	$\mu\text{g}$	
		no colostrum											
0	.	384	116	190	69	18	158	217	249	180	.	77	
1	2	226	71	63	96	80	40	50	25	62	46	21	
2	2.7	...	146	104	115	173	20	14	2	17	14	40	
3	3.3	187	203	114	...	413	11	0	x dead	4	14	4	
4	3.6	.	.	101	...	353	3 x	8	x dead	x dead	8	x dead	
5	3.9	197	.	104	283	.	7	x 300	.	.	.	.	
6	4.2	155	.	dead	.	.	5	22	.	.	*	.	
7	4.4	.	.	.	...	.	x 25	.	.	.	*	.	
8	4.6	.	.	.	.	.	25	22 F	.	.	130	recovered	
9	4.8	.	.	.	124	.	.	58 F	.	.	.	.	
10	.	.	.	.	.	.	8	34 F	.	.	.	.	
		.	.	.	.	.	x dead	69 F	.	.	.	.	
11	.	.	.	.	.	.	.	x dead	.	.	.	.	

x One thiamine injection given during this week.

F During this period calf 17 scoured a great deal and these high values are probably a reflection of fecal contamination which was not entirely prevented by the feces bags used.

\* Thiamine given orally at each feeding this week.

toms set in and those obtained after the animals were cured. In the case of the control calves, average values are given.

In all cases of scouring, the first treatment used was oral sulfathalidine, and whenever there was any evidence of respiratory disturbance, sulfamerazine was given. Neither of these treatments had any apparent effect on the course of the deficiency, although they have been effective in correcting the disturbances for which they were given in cases involving the positive control calves.

TABLE 3

*Blood and urine pyruvate on calves fed thiamine-low rations as compared to calves on the control ration.*

CALF NO.	URINARY PYRUVATE, MG/DAY AS PYRUVIC ACID		BLOOD PYRUVATE, $\mu$ G/ML AS PYRUVIC ACID	
Deficient calves				
	<i>Before thiamine injection</i>	<i>After thiamine injection</i>	<i>Before thiamine injection</i>	<i>After thiamine injection</i>
16	107	44	.	.
17	95	39	67	25
31	65	died	20 <sup>1</sup>	..
32	46 <sup>1</sup>	died	24 <sup>1</sup>	
36	75	32	45	21
39	64	died	24	
Positive control calves (average values)				
24		43		26
26		27		22
30		32		19
33		23		19
40		34		21

<sup>1</sup> Average values.

#### SUMMARY AND CONCLUSIONS

Fourteen new-born dairy calves were used in a study of their thiamine requirements. Thirteen of these had colostrum for from 12 to 48 hours and one had no colostrum. All received the highly purified diet previously used in these studies. Seven of the calves served as positive controls and 7 as test animals; the only difference in the treatment of the 2 groups was that

the positive controls received thiamine, whereas the test group did not. Records of daily observations for signs of deficiency, weekly weights, and weekly examination of 24-hour urine samples and of blood for thiamine and pyruvic acid were made for 11 weeks, or for as long as the calves lived. From the data obtained, the following conclusions seem justified:

1. The bovine species requires thiamine, supplied either in the diet or by the symbiotic action of rumen or intestinal microorganisms.

2. On a thiamine-low diet the calf develops pathological signs, urinary excretion usually reaches a level below 10  $\mu$ g per day, and blood and urinary pyruvate levels are elevated above normal.

3. Thiamine deficiency in the young dairy calf is characterized by weakness, incoordination of legs, convulsions and head retraction, and, in some calves, by severe scouring, anorexia and dehydration.

4. The convulsions and typical polyneuritic symptoms respond almost immediately to thiamine therapy. However, in all cases of severe dehydration, the calves died, even though thiamine was administered.

#### ACKNOWLEDGMENTS

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The thiamine analyses were carried out by Shirley Spaeth, and the pyruvate determinations by Barbara Chase.

Thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, calcium pantothenate, biotin, nicotinic acid, ascorbic acid and  $\alpha$ -tocopherol acetate were generously supplied by Hoffmann-La Roche, Inc., Nutley, New Jersey, through the courtesy of Dr. J. C. Bauernfeind. Pteroylglutamic acid was supplied by Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, through the courtesy of Dr. E. L. R. Stokstad. Choline and experimental choline dry mix were supplied by Merck and Company, Inc., Rahway, New Jersey. Inositol was supplied by the Corn Products Refining Company, New York. Vitamin A and D capsules were supplied by the Gelatin Products Corporation, Detroit, Michigan.

Sulfathalidine (phthalylsulfathiazole) was generously supplied by Sharpe and Dohme, Philadelphia, Pennsylvania, through the courtesy of Dr. S. F. Scheidy.

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# UNIDENTIFIED FACTORS ESSENTIAL FOR GROWTH AND HEMOGLOBIN PRODUCTION IN FOXES<sup>1</sup>

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## TWO FIGURES

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## INTRODUCTION

In our original studies on the nutritional requirements of the fox, the importance of folic acid was clearly established (Schaefer et al., '47), and preliminary results indicated that fresh liver contains an unknown factor essential for this species. When fox pups or adults were fed a purified ration containing all the known crystalline vitamins, including folic acid, adult animals maintained body weight for 32 to 40 weeks and weanling animals gained weight for 15 to 20 weeks, before showing general failure and sudden death. The deficiency syndrome could be alleviated or prevented by supplementing the ration with fresh raw liver or raw whole milk. Further evidence that foxes require an unidentified factor (or factors), which is distinct from the known crystalline vitamins, is presented here.

<sup>1</sup>Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was reported briefly in *Fed. Proc.*, 6: 420, 1947. These studies were supported by project 614, Wisconsin Agricultural Experiment Station.

## EXPERIMENTAL

Five adult silver foxes and 15 silver, platinum silver, and red fox pups from 9 different litters were used in this experiment. The basal ration had the following percentage composition: sucrose 66, casein <sup>2</sup> 19, cottonseed oil 8, cod liver oil 3 and salts IV (Phillips and Hart, '35) 4. Each 100 gm of ration was supplemented with 0.2 mg thiamine chloride, 0.2 mg pyridoxine hydrochloride, 0.4 mg riboflavin, 1.5 mg calcium pantothenate, 4.0 mg niacin, 100 mg choline chloride, 0.1 mg folic acid, 0.025 mg biotin, 25 mg i-inositol, 50 mg p-aminobenzoic acid, 2 mg  $\alpha$ -tocopherol and 0.5 mg 2-methyl-1,4-naphthoquinone. The general experimental procedures were the same as those reported previously (Schaefer et al., '47). When the animals showed deficiency symptoms indicated by a 15 to 30% loss in body weight, reduction in hemoglobin, depigmented matted fur, anorexia and in some cases paralysis, they were fed the supplements shown in table 1. If an animal failed to show a response after administration of the supplement for 4 weeks, the material as fed was considered to be inactive. In some animals failure was rapid, and where death appeared imminent it was necessary to administer additional therapy prior to completion of the 4-week period.

Although variations in red and white blood cell counts were observed, no particular trend was apparent. At the time of the deficiency just prior to administration of therapy the total leucocyte count averaged 7500 per mm<sup>3</sup> and the ratio of neutrophils to lymphocytes averaged 56% to 45%, respectively. After feeding active supplements to the deficient animals the total leucocyte count averaged 8800 per mm<sup>3</sup> and the neutrophils were reduced to an average of 30% with a proportionate increase in the number of lymphocytes to 67%.

Figures 1 and 2 illustrate typical curves for the body weight changes of animals developing a deficiency and showing subsequent response to therapy. Depigmented matted underfur was observed in all foxes receiving only the basal ration.

<sup>2</sup> Smaco vitamin test.

TABLE 1  
The effect of feeding various supplements to foxes developing a deficiency of the unidentified factor(s).

FOX NO.	RATION	TOTAL DAYS ON SUPPLEMENT PRIOR TO SUPPLEMENT	SUPPLEMENT	DAYS AFTER SUPPLEMENT	BODY WT. CHANGE AFTER SUPPLEMENTATION	HEMOGLOBIN		NEUTROPHILES/LYMPHOCYTES	
						Before	After	Before	After
						gms %	gms %	%	%
13 ♀	Basal	210	10% fresh raw liver	28	+ 0.6	13.3	18.8	37/61	35/65
22 ♂	Basal	336	1% methanol extract <sup>1</sup>	35	+ 0.9	13.3	16.8	70/30	33/67
10 ♂	Basal	136	10 ml Sharpe and Dohme liver extract <sup>3</sup>	14	— 0.1	12.8	12.6	68/31	51/46
10 ♂	Basal + double vitamin level	157	10% fresh raw liver	56	+ 2.7	12.6	16.9	51/46	45/55
9 ♂	Basal	131	30 ml Sharpe and Dohme liver extract <sup>3</sup>	56	— 1.9	17.3	16.5	46/54	70/39
9 ♂	Basal	187	1% methanol extract <sup>1</sup>	56	+ 1.3	16.5	17.7	70/29	27/70
18 ♀	Basal	175	6% dried brewers' yeast	70	+ 2.0	13.2	13.5	78/21	21/79
18 ♀	Basal + 6% yeast	245	50 ml Sharpe and Dohme liver extract <sup>3</sup>	53	0	13.5	13.9	21/78	21/78
18 ♀	Basal + 6% yeast	298	1% methanol extract <sup>1</sup>	26	0	13.9	16.5	21/78	14/86
11 ♀	Basal	224	6% dried brewers' yeast	35	0	14.3	14.5	14/86	31/67
11 ♀	Basal + 6% yeast	259	1% methanol extract <sup>1</sup>	28	0	14.5	16.1	31/67	43/57
40 ♂	Basal <sup>2</sup>	357	6% residue <sup>3</sup>	50	— 0.5	14.9	16.8	58/40	51/46
40 ♂	Basal + 6% residue	427	4% residue <sup>3</sup> + 2% methanol extract <sup>6</sup>	56	+ 0.8	14.5	17.7	20/80	42/56
35 ♂	Basal + double the vitamin level	285	10% fresh raw liver	56	+ 2.7	15.2	18.8	43/54	21/77
21 ♀	Basal + double the vitamin level + ascorbic acid 50 mg/day	98	10% fresh raw liver	63	+ 2.7	11.8	16.2	33/67	16/82
26 ♀	Basal + 3% whole liver substance <sup>6</sup>	112	6% whole liver substance <sup>6</sup>	49	+ 1.3	14.7	16.8	19/81	31/62
33 ♂	Basal + 2% "1:20" L.E.P. <sup>7</sup>	364	3% "1:20" L.E.P. <sup>7</sup>	42	+ 1.3	16.2	17.0	42/55	40/80
34 ♂	Basal + 2% "1:20" L.E.P. <sup>7</sup>	344	3% "1:20" L.E.P. <sup>7</sup>	21	— 0.2	13.1	12.3	46/48	45/48
34 ♂	Basal + 3% whole liver substance <sup>6</sup>	365	Raw whole milk 50 ml/day	35	+ 1.7	12.3	16.7	45/48	28/70
2 ♂	Basal + 3% whole liver substance <sup>6</sup>	154	10% fresh raw liver (removed whole liver substance)	84	+ 2.5	11.4	16.5	61/30	42/55
6 ♂	Basal + 6% whole liver substance <sup>6</sup> + ascorbic acid 50 mg/day	140	Raw whole milk, 50 ml/day	70	+ 1.8	10.4	15.8	66/32	40/58
14 ♀	High protein (30% casein)	210	1% methanol extract <sup>1</sup>	28	+ 0.5	14.5	17.0	53/41	34/66

<sup>1</sup> Methanol soluble fraction of fresh liver, 1 ml equivalent to 10 gm fresh liver.

<sup>2</sup> Animals recovered from previous folic acid deficiencies.

<sup>3</sup> Sharpe and Dohme — refined liver extract no. 2505, 1 ml per day injected intramuscularly.

<sup>4</sup> Adult animals.

<sup>5</sup> Residue of fresh liver (methanol insoluble fraction).

<sup>6</sup> Wilson's whole liver substance.

<sup>7</sup> Wilson's "1:20" liver extract powder.



Those fed supplements of fresh liver or dried liver powders had well-developed prime pelts. Autopsy of the 7 animals which died of the deficiency (table 2) revealed yellow, enlarged, fatty livers and degenerate pale-colored kidneys.

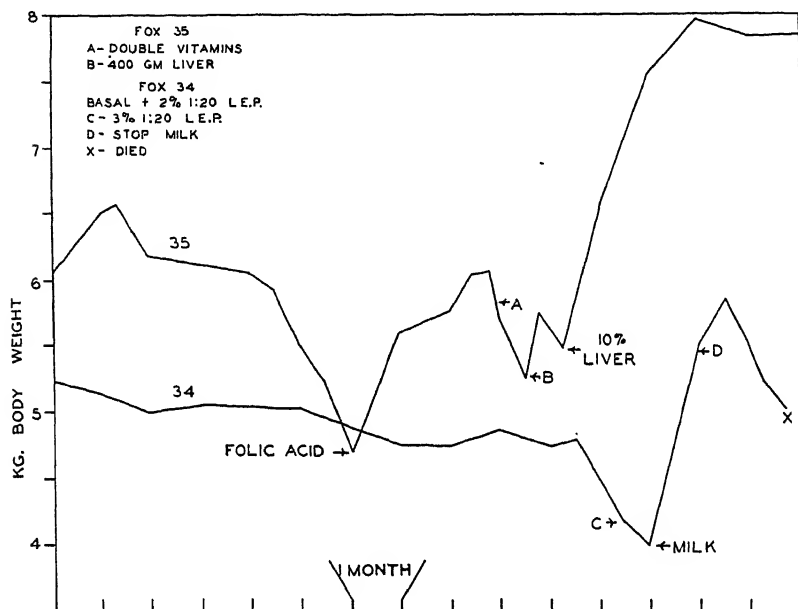


Fig. 1 Body weight curves of adult foxes 34 and 35. Fox 35 was started on the basal ration (without folic acid); folic acid therapy was started at 0.5 mg per day and continued to point A; A = fed the basal ration plus double the vitamin supplement until point B; B = started the basal ration and 400 gm of fresh beef liver fed over a period of 1 week. Fox 34 was started on the basal ration minus folic acid plus 2% "1:20" liver extract powder; folic acid included in the ration after the animal was on experiment 5 months; when the milk supplement (50 ml/day) was started "1:20" liver extract powder was discontinued.

To eliminate the possibility that fresh liver or milk merely supplied additional amounts of the known crystalline vitamins, or of protein, the following supplementations were made: Foxes 10, 21 and 35 received daily twice the amount of the vitamin supplement per 100 gm of ration. Fox 8 was fed 30% casein instead of 19%, in addition to the double level of vita-

mins. Foxes 6, 7 and 21 received 50 mg ascorbic acid per 100 gm ration. In all animals these supplements neither alleviated nor prevented the deficiency syndrome.

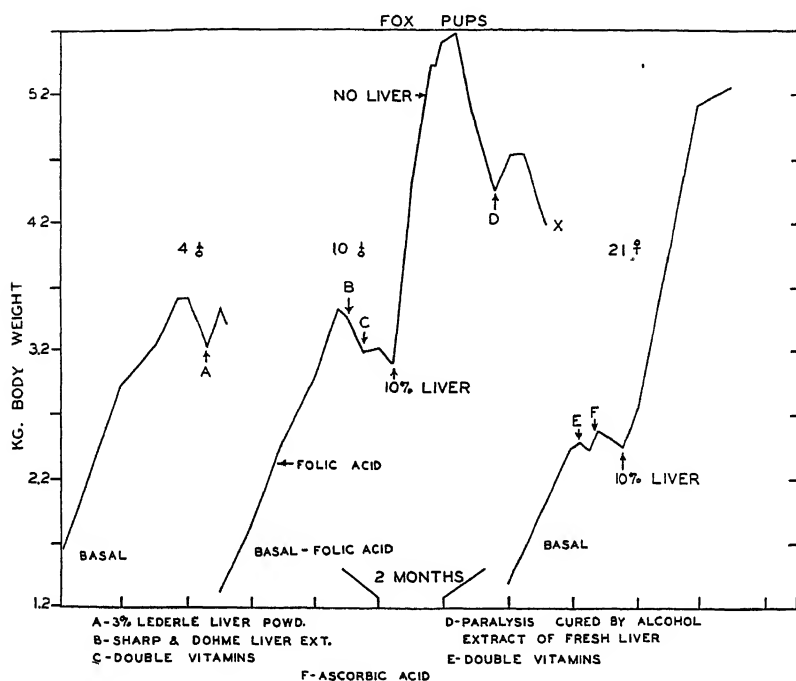


Fig. 2 Body weight curves of fox pups 4, 10 and 21. Fox 4 died after a slight response to Lederle liver powder. Fox 10: B = 10 ml of Sharpe and Dohme liver extract no. 2505 injected intramuscularly; D = paralysis cured by the oral administration of 15 ml of methanol extract and 4 ml intraperitoneally over a period of 1 week; therapy was discontinued as the animal recovered from the paralysis; X = died. Fox 21: E = started feeding twice the vitamin supplement given in table 1; this was discontinued when 10% fresh liver was added to the ration; F = started 50 mg ascorbic acid per 100 gm of ration.

The results with foxes 2, 6, 26, 33 and 34 indicate that the whole liver substance<sup>3</sup> fed at 3 to 6%, and "1:20" liver extract powder fed at 2 to 3% did not contain the activity supplied by 10% fresh liver and that the potency varies in different samples of the same product.

<sup>3</sup> Wilson's.

Since most of the deficient animals had a suboptimal hemoglobin concentration, a refined liver extract of the antiper-nicious anemia factor was administered to foxes 9, 10 and 18. The results indicate that at the levels of 10, 30 and 50 ml, injected intramuscularly (2 ml every other day), the extract did not stimulate hemoglobin regeneration.

TABLE 2  
*Animals that died of the liver factor deficiency.*

FOX NO.	TOTAL DAYS ON EXP'T.	RATION AND SUPPLEMENT PRIOR TO DEATH	LOSS OF BODY WEIGHT	HEMOGLOBIN AT TIME OF DEATH	NEUTROPHILES/LYMPHOCYTES	LIVER ANALYSIS	
						fat wet basis	H <sub>2</sub> O
			kg	gm %	%	%	%
3 ♂	145	Basal	0.2	7.2	49/51	34.5	54.9
10 ♂	287	Basal <sup>1</sup>	0.5	15.7	55/42	..	...
34 ♂ <sup>2</sup>	390	Basal <sup>3</sup>	0.9	15.7	27/65	13.3	67.3
38 ♂ <sup>2</sup>	294	Basal + 0.4 mg folic acid per 100 gm ration	0.8	16.6	75/25	..	..
7 ♀	121	Basal + ascorbic acid	1.0	16.3	45/36	19.2	60.3
8 ♀	252	High protein ration + double vitamin level	0.3	17.0	71/28	..	..
4 ♂	142	Basal + 3% liver preparation <sup>4</sup>	0.2	9.2	72/27	11.7	72.9

<sup>1</sup> After 1 response to fresh liver, the supplement was withdrawn from the ration.

<sup>2</sup> Adult foxes.

<sup>3</sup> After 1 response to raw whole milk the supplement was withdrawn from the ration.

<sup>4</sup> Lederle.

In a search for other sources of the liver factors, the ration for foxes 18 and 11 was supplemented with 6% dried brewers' yeast. At the time of therapy, fox 18 had lost 1 kg of body weight, hemoglobin was 13.2 gm % and the neutrophile-lymphocyte ratio was 78/21. Eight weeks after feeding yeast, the body weight had increased from 3.3 to 5.3 kg and the neutrophile-lymphocyte ratio had returned to a normal of 21/79 for foxes. However, supplementation of the ration with yeast

or yeast plus 50 ml refined liver extract no. 2505<sup>4</sup> (injected intramuscularly) failed to correct the anemia. The ration for fox 11 was supplemented with 6% yeast at the time the hemoglobin value had decreased to a suboptimal level with body weight normal. No response in hemoglobin formation was noted, but normal body weight was maintained.

These observations indicate that 2 or more unidentified factors are supplied by fresh liver, one of which is required for maintenance of body weight (present in yeast or liver) and the other for optimum hemoglobin concentration.

The studies of Jaffe and Elvehjem ('47), on the fractionation of the growth-stimulating factor in liver for rats on a yellow corn-soybean meal ration, offered the possibility of following the activity of similarly prepared concentrates for foxes. Extracts of fresh liver were prepared as follows: 10 kg of fresh beef liver was ground in a meat grinder and thoroughly stirred for 45 minutes with 10 liters of absolute methanol. The mixture was filtered by means of a canvas cloth and filter press. The residue was extracted twice with 10 liters of 60% methanol and filtered. The combined filtrates were concentrated under reduced pressure (35 mm Hg) to a volume of 2 liters. The filtrate was then extracted twice with equal volumes of ether. The ether soluble fraction was discarded and the filtrate concentrated under vacuum to approximately 1 liter; pH was adjusted to 6.8; the solids were removed by filtration and discarded. The volume of the solution was adjusted to 1 liter so that 1 ml of extract was equivalent to 10 gm of fresh liver. Total solids of the extract were 0.31 to 0.35 gm per milliliter. This preparation was fed at a level of 1 ml per 100 gm of ration (which is referred to as 1% methanol extract). The residue of fresh liver, after the methanol extraction, was dried at 34°C. to 40°C. for 30 hours and finely ground.

The activity of the methanol extract was measured on animals 9, 11, 14, 18 and 22. At the time of the deficiency syndrome, when anorexia and paralysis were evident in pups 9,

<sup>4</sup>Sharpe and Dohme.

14 and 22, 3 ml of methanol extract was administered orally and 1% added to the ration thereafter. The hemoglobin level was increased to an optimum level in all the animals. Foxes 9 and 22 gained 1300 and 900 gm, respectively, but failed to attain their previous normal body weight. After this initial response in body weight to 1% methanol extract, the supplement was increased to 2% and finally 3%. The animals again showed a loss of body weight. Further supplementation of the ration for fox 9 with 4% residue plus 1% methanol extract resulted in a gain of 900 gm body weight. Fox 14 was sacrificed for its pelt after the initial response to the methanol extract, thus excluding additional observations as to whether this preparation contained only a portion of the activity of fresh liver. When the feeding of yeast to foxes 18 and 11 did not alleviate the anemia, their rations were supplemented with 1% methanol extract. Within a period of 3 to 4 weeks the hemoglobin level increased from 13.9 to 16.5 gm % in fox 18 and from 14.5 to 16.1 gm % in fox 11.

When the first decline in body weight was noted in fox 40, the ration was supplemented with 6% residue (methanol insoluble fraction of fresh liver), but loss in body weight continued. When anorexia developed the ration supplement was changed to 4% residue plus 2% methanol extract. After 4 weeks on this regimen body weight had increased to the previous normal value and hemoglobin increased from 14.5 to 17.7 gm %.

#### DISCUSSION

Fresh raw liver and raw whole milk are good sources of the unidentified factors required for the normal nutrition of foxes. Yeast apparently contains 1 factor which is essential for growth and maintenance of body weight, but is lacking or deficient in a factor necessary for the prevention of anemia.

Preliminary evidence indicates that the methanol soluble fraction of fresh liver contains an active factor(s) which is essential for optimum hemoglobin concentration and normal body weight. A comparison of the animals receiving the

methanol extract with foxes 9 and 40, which received the extract plus the methanol insoluble residue indicates that at least 2 factors are present in fresh liver. Since the antipernicious anemia liver extract failed to stimulate hemoglobin formation in the deficient foxes, the present unidentified factor(s) apparently is not related to the dog anemia factor reported by Ruegamer et al. ('47). The similarity between the monkey anti-anemic factor reported by Cooperman et al. ('45, '46), and the unidentified fox factor(s) is indicated by the general blood changes, loss in body weight and response to similar crude materials.

#### SUMMARY

1. Fox pups and adults require an unidentified factor(s), distinct from the known crystalline vitamins, for normal nutrition.
2. The deficiency syndrome produced by a lack of this factor(s) is characterized by anorexia, loss in body weight, depigmented, matted underfur, suboptimal hemoglobin levels, reversal of the neutrophile-lymphocyte ratio, fatty, light-colored livers and death.
3. Supplementation of the ration with 10% fresh raw liver or 50 ml per day of raw whole milk corrected the deficiency symptoms.
4. Preliminary evidence indicates that there may be more than 1 factor in fresh liver required by the fox.

#### ACKNOWLEDGMENTS

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# THE REQUIREMENT OF UNIDENTIFIED FACTORS FOR MINK<sup>1</sup>

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## ONE FIGURE

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## INTRODUCTION

In previous studies on the nutritional requirements of mink (Schaefer et al., '46) it was shown that when these animals were maintained on purified rations supplemented with all the known crystalline vitamins (including folic acid), a deficiency syndrome occurred characterized by anorexia, loss in body weight, suboptimal hemoglobin levels, paralysis and improper furring followed by sudden death. The feeding of fresh raw liver or raw whole milk prevented or alleviated the deficiency. The requirement of unidentified factor(s) for mink is similar to that observed in foxes (Schaefer et al., '47). In this paper we wish to present data on the requirement and fractionation of the unidentified factors for adult mink and kits.

## EXPERIMENTAL

Thirteen adult mink and 40 kits were used in these studies. The housing, care and handling of the mink have been described (Schaefer et al., '46). The composition of the purified basal ration (diet I) employed is the same as that used in the

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fox studies (Schaefer et al., '47). A number of animals were fed a high protein ration, where casein was increased to 30% at the expense of sucrose (diet II), and 10% gelatin added at the expense of sucrose (diet III).

### *Studies with adult mink*

Adult mink receiving the purified rations developed deficiency symptoms characterized by loss in body weight, depigmented and poor quality fur, suboptimal hemoglobin levels and anorexia, in periods of 132 to 259 days. The data presented in table 1 show the response in body weight gain and increase in hemoglobin concentration upon addition of various supplements.

Hemoglobin determinations were made on 7 deficient animals before supplementation and again 8 weeks after. Six animals showed suboptimal levels ranging from 10.7 to 15.3 gm %, which increased 3.0 to 5.6 gm % upon the feeding of fresh raw liver or raw whole milk. A suboptimal hemoglobin concentration of 15.3 gm % was also observed in mink 7 and 21, whereas animals 9 and 16 had levels of 19.0 and 16.7 gm %, respectively. Total red and white blood cell counts were not altered in a specific trend. It should be emphasized that occasionally animals died very suddenly without the usual forewarning of anorexia, paralysis and lowered hemoglobin. Adult mink receiving only the purified rations died, and autopsy revealed severe fatty degeneration of livers and kidneys.

The results with mink 2, 7, 8 and 11 indicate that liver fraction L,<sup>2</sup> and "1:20" liver extract powder fed at 2%, and whole liver substance fed at 3%, did not contain as much of the unknown factor(s) as is supplied by 10% fresh liver. In these animals the time of onset of the deficiency syndrome was about 38 weeks, in comparison with the average time of 25 weeks for mink receiving only the purified rations without liver supplements. The results with mink 6 and 19 indicate

<sup>2</sup> Wilson's.

TABLE 1  
*Studies on the unidentified factor required by adult mink.*

MINK NO.	RATION	INITIAL WT.	TOTAL DAYS ON EXP. <sup>1</sup> PRIOR TO SUPPLEMENT	SUPPLEMENT AT THE TIME OF DEFICIENCY SYNDROME	DATA BEFORE SUPPLEMENT AND 8 WKS. AFTER			
					Body wt.		Hemoglobin	
					Before	After	Before	After
		gm			gm	gm	gm %	gm %
9 ♀ <sup>1</sup>	Diet I	860	259	None (died)	410	...	19.0	...
21 ♂	Diet I	1020	161	None (died)	800	...	15.3	...
11 ♀	Diet I + 3% W.L.S. <sup>2</sup>	600	260	Milk ad lib. for 7 days	430	565	...	...
			325	6% W.L.S. <sup>2</sup>	520	585	...	...
20 ♂	Diet I + 6% W.L.S. <sup>2</sup>	1230	286	None (no deficiency syndrome)	1250	...	19.0	...
22 ♂	Diet II	1070	161	10% fresh liver	735	1110	14.9	17.9
16 ♀ <sup>1</sup>	Diet II	600	168	Milk ad lib.	475	625	16.7	17.4
17 ♂ <sup>1</sup>	Diet II	1120	372	None (died)	830	...	...	...
1 ♂ <sup>1</sup>	Diet III	1130	132	10% fresh liver	750	1140	10.7	16.3
6 ♀ <sup>1</sup>	Diet III	660	216	10% fresh alfalfa	645	570	17.3	13.7
			272	20% fresh liver	570	650	13.7	17.3
19 ♀ <sup>1</sup>	Diet III	640	154	Fresh tomato extract <sup>3</sup>	540	500	15.5	15.3
			210	10% fresh liver	500	660	15.3	19.4
2 ♂	Diet III + 2% "1:20" <sup>1</sup> L.E.P. <sup>4</sup>	990	259	Milk ad lib.	740	1120	13.5	17.5
7 ♀	Diet III + 2% fraction "L" <sup>5</sup>	650	315	None (died)	550	...	15.3	...
8 ♂	Diet III + 2% fraction "L" <sup>5</sup>	1110	231	10% fresh liver	900	1250	14.5	17.9

<sup>1</sup> Animals which had a previous folic acid deficiency.

<sup>2</sup> Wilson's whole liver substance.

<sup>3</sup> Filtrate of fresh ground tomatoes fed at a level equivalent to 50 gm fresh tomatoes per day.

<sup>4</sup> Wilson's "1:20" liver extract powder.

<sup>5</sup> Wilson's fraction "L" liver extract.

that fresh tomatoes and alfalfa, at the levels fed, were inactive in alleviating the deficiency. Mink 17, receiving diet II, did not exhibit a severe loss of body weight; however, erratic body weight changes and depigmented matted underfur were noted after approximately 100 days on experiment. The animal died suddenly after being on experiment for 372 days.

### *Studies with mink kits*

The mink kits were weaned at 8 to 9 weeks of age and fed the basal ration supplemented with 20 gm of stock ranch meat ration for 3 days. After this period the animals were weighed and placed on the purified diets. Weight of the kits at the start of the experiment varied from 325 to 415 gm for females and from 395 to 650 gm for males.

The survival time of all mink fed diets I or II without further supplementation varied from 34 to 325 days. Average data for the deficient animals and for the controls receiving supplements of fresh liver or milk are shown in table 2.

TABLE 2

*Mink kits receiving purified rations with and without liver supplements.*

RATION	TOTAL DAYS ON EXP'T.	BODY WEIGHT	HB	LIVER ANALYSIS		
				fat wet basis	H <sub>2</sub> O	
		gm	gm %	%	%	
Mink kits which died of the deficiency.						
Average of 4 (♂)	Diet I	260	807	17.4	48.6	38.0
Average of 3 (♀)	Diet I	54	405	14.8 <sup>1</sup>	...	...
Mink no. 65 (♂)	Diet II	210	755	...	18.5	76.0
Mink kits — controls receiving liver or milk supplement						
Average of 5 (♂)	Diet I + 20% fresh liver	260	1210	19.8	8.6 <sup>2</sup>	61.3 <sup>2</sup>
Average of 3 (♀)	Diet I + 20% fresh liver	56	700	18.1	5.8	66.8
Mink no. 69 (♀)	Diet I + 25 ml raw whole milk per day	56	650	17.7	...	...

<sup>1</sup> Hemoglobin determined on 1 ♀.

<sup>2</sup> Liver fat and moisture analysis on 1 male.

Severe, fatty, yellow-colored, enlarged livers and pale-colored kidneys were observed in kits which died of the deficiency. Kits receiving the purified rations developed a depigmented, matted underfur. Once this improper furring developed it was not possible to produce a black, densely furred pelt by feeding sources of the liver factor(s); however, improvement in the general texture of the fur was noted. That this lack of pigmentation was not a genetic mutation was established by maintaining these animals on the purified ration supplemented with liver throughout the shedding cycle. The new underfur and guard hair were of good texture and black in color.

The responses in body weight and hemoglobin concentration of some of the mink when various supplements were fed are presented in table 3. The results with mink 31 receiving 10% fresh liver indicated that the requirement of the liver factor(s) was greater for kits than for adults. Gain in body weight was very rapid for 56 days, but this was followed by general failure and loss of body weight. When the level of fresh liver was increased to 20% of the ration, a sustained increase in body weight resulted until maturity was reached. A similar response was obtained with female no. 64.

Whole liver substance<sup>3</sup> fed at 3% and 6% gave results similar to those with adult mink. Fresh beef pancreas failed to produce a response in mink 62, but in another male (mink 58) a slight increase of 85 gm body weight occurred in 28 days. The activity of fresh raw milk is illustrated in the case of mink 45, which received the supplement of 25 ml per day added to the water. The same response was observed with mink 69. Both of these animals showed good growth, a good fur coat and a normal blood picture. Upon autopsy, however, degeneration in both the liver and kidney was noted. The activity of fresh raw whey is indicated in the case of mink 48. Dried brewers' yeast fed at a 6% level to kits 33 and 38 resulted in a gain in body weight; but body weight was not maintained. The same methanol extracts of fresh liver that were found to be active for foxes (Schaefer et al., '47) were

<sup>3</sup> Wilson's.

TABLE 3  
*Response to supplements administered to mink kits at the time of deficiency symptoms.*

MINK NO.	RATION	SUPPLEMENT ASSAYED	TIME INTERVAL			BODY WT.		HEMOGLOBIN	
			Before supplement or change in supplement	After supplement	Days interval	Before	After	Before	After
			Total days on exp.			gm	gm	gm %	gm %
55 ♀	Diet I	20% fresh liver	56	42	14	820	1000	18.8	...
31 ♂	Diet I + 10% fresh liver	20% fresh liver	63	98	35	375	500	14.8	19.2
24 ♂	Diet I	6% W.L.S. <sup>1</sup>	102		11	775	1320	15.5	19.6
62 ♂	Diet I	20% fresh liver	168		133	965	1250	...	19.3
45 ♀	Diet I	20% fresh beef pancreas	94		70	660	500	16.7	15.8
48 ♂	Diet I	20% fresh liver	105		84	500	1200	15.8	18.9
38 ♀	Diet I	Raw whole milk	63		21	400	800	14.6	18.4
	Diet I	Raw whole whey <sup>2</sup>	119			665	945	...	...
	Diet I	6% dried brewers' yeast	126			550	670	...	17.6
		6% yeast + 2% methanol extract <sup>3</sup>	161		28	585	705	17.6	20.0
39 ♂ <sup>4</sup>	Diet I	4% residue <sup>2</sup>	220		42	725	885	19.7	...
		8% residue	278		7	775	700	...	...
		4% residue + 1% heated methanol extract <sup>3</sup>	285		28	700	835	...	...
67 ♂	Diet I	Double amount of vitamin supplement	210		112	855	700	16.9	16.7
		1% heated methanol extract	322		14 <sup>1</sup>	700	775	16.7	17.0

<sup>1</sup> Wilson's whole liver substance.

<sup>2</sup> Raw whole whey fed at equivalent of 50 ml whole milk.

<sup>3</sup> Methanol soluble fraction of fresh liver, 1 ml equivalent to 10 gm fresh liver.

<sup>4</sup> Animal having a previous folic acid deficiency.

<sup>5</sup> Residue of fresh raw liver (methanol insoluble fraction).  
<sup>6</sup> 50 ml methanol extract autoclaved at 15 lbs. pressure for 15 minutes, neutralized and filtered. Total solids = 0.15 gm per ml.

<sup>7</sup> Animal accidentally killed.

used in these studies. After mink 33 showed anorexia for a period of 3 days, 2 ml of methanol extract of fresh liver was fed orally and 1% (1 ml equivalent to 10 gm fresh liver per 100 gm of ration) mixed into the ration thereafter. Three weeks later body weight had increased from 564 to 725 gm, and increasing the methanol extract to 2% resulted in an additional 75 gm weight gain. Mink 38, after responding to

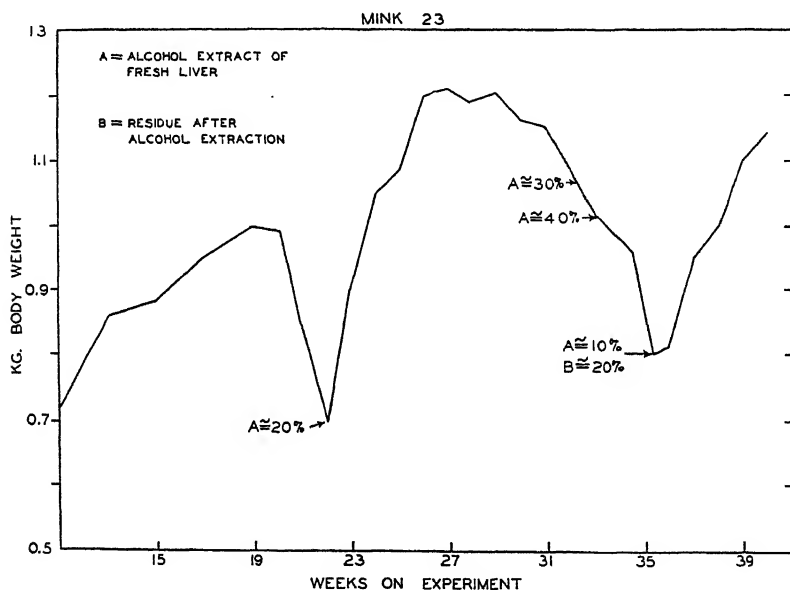


Fig. 1 Body weight curve of mink 23, showing the response in body weight gain, first to the methanol soluble fraction, and then to the methanol insoluble fraction of fresh raw liver.

6% dried brewers' yeast, again lost body weight and the additional symptom of paralysis of the rear quarters was noted. The oral administration of 2 ml methanol extract for the first 3 days of therapy, and supplementing the ration with 2% thereafter, cured the paralysis and the animal gained 120 gm weight.

Preliminary evidence indicating the existence of 2 separate active fractions in fresh raw liver was obtained with animals 23, 39 and 44. Figure 1 shows the body weight curve of mink

23. The administration of 2% methanol extract of fresh liver (equivalent to 20% fresh liver) produced a dramatic increase of 500 gm body weight within 4 weeks. When loss in body weight again became evident the level of methanol extract fed was increased to 3%, and finally 4%. Loss in body weight continued and after anorexia had occurred for 3 days, 2 gm of the residue of liver (methanol insoluble material) was fed orally in a watery paste for 4 days. It was then mixed into the ration at an 8% level, which was approximately equivalent to 20% fresh liver, along with 1% of the methanol extract. The resulting response as shown by the gain in body weight indicated that at least 2 factors were involved. Similar results were obtained with mink 39, except that the feeding of the 2 fractions was reversed. As the first deficiency became apparent the residue fraction was fed at a 4% level. Gain in body weight was again temporary and was not sustained by increasing the residue to 8%. The second deficiency, characterized by loss in body weight, paralysis of the rear quarters and anorexia, occurred 65 days after the residue therapy had been started. The ration was then supplemented with 1% of an autoclaved (15 pounds of pressure for 15 minutes) methanol extract plus 4% residue. Anorexia and paralysis were alleviated and gain in body weight resulted. As the first sustained loss in body weight was noted in mink 44 the diet was supplemented with 8% residue, and this produced a slight gain of 90 gm weight. Fifty-five days after this continued therapy the animal died suddenly. Mink 67 (table 3) receiving the basal ration plus twice the vitamin supplement developed anorexia, paralysis and loss of body weight. The ration was then supplemented with 1% heated methanol extract. Anorexia and paralysis were alleviated and body weight increased. Two weeks after initiation of therapy the animal was accidentally killed.

#### DISCUSSION

It is evident from these data that mink are unable to survive upon a purified diet containing sucrose, casein, mineral salts,

cottonseed oil, cod liver oil and all the known crystalline vitamins. The maintenance or gain of body weight in adult and young growing mink is only temporary and once the stores in the body are depleted, nutritive failure and death result. Suboptimal hemoglobin levels (mainly noted in adults), the grayish-matted, dull-appearing fur, and paralysis of the hind quarters, are outward indications of upset metabolism. The length of time elapsing between the start of the experiment and a severe loss in body weight or death varied in adults from 132 to 259 days and for kits from 34 to 325 days. The average survival time for 24 mink kits was 140 days. It should be pointed out again that each mink should be considered individually with a recognition of the genetic factors which may influence the experiment.

During the course of these experiments it became evident that if mink kept on the purified diets were to be saved from certain death, it was necessary to administer fresh raw liver or raw whole milk. Further studies indicated that 6% dried brewers' yeast contains part of the activity furnished by fresh liver. The animals responded to yeast, as noted by a gain in body weight, but when they were maintained on this regimen failure again occurred. The observation reported in the fox studies (Schaefer et al., '47) that the methanol soluble fraction of fresh raw liver was active in correcting this second deficiency was tried and found successful in mink.

Preliminary observations indicate that several factors required by mink are present in fresh raw liver. Initial separation of the activity in fresh raw liver by methanol extraction indicates that the methanol soluble fraction contains 1 factor or factors and that the methanol insoluble fraction contains another factor or factors.

#### SUMMARY

1. Mink kits and adults fed highly purified rations supplemented with all the known crystalline vitamins require additional unidentified factors for normal nutrition. The characteristic deficiency symptoms are described.



2. Preliminary evidence from the fractionation of the unidentified factors in fresh raw liver indicates that methanol extraction removes 1 factor(s) and that the insoluble residue contains another factor(s).

#### ACKNOWLEDGMENTS

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## SUPPLEMENTAL VALUE OF CERTAIN AMINO ACIDS FOR BEEF PROTEIN

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Beef is an important source of animal protein in the American dietary. In 1940 it supplied 41% of the protein in all kinds of meat, including meat by-products, produced in the United States (Rose et al., '42). In 1944-45, meat, including poultry and fish, supplied 28% of the protein contributed by the major food groups consumed in this country (Clark et al., '47).

The protein in beef is generally considered to be highly digestible and of relatively high biological value, but there is some evidence indicating that beef may be deficient in certain amino acids. For example, Mitchell and Smuts ('32) conducted paired feeding tests with young rats to determine the effects of the addition of 0.25% cystine to diets containing 8 and 20%, respectively, of beef protein. The addition of cystine to the diet containing 8% protein induced significantly better growth than the control diet, but the addition of cystine to the diet containing 20% protein was without material effect.

In a recent paper Mitchell and Block ('46) present a comparison of the amino acid content of various proteins, with egg as a standard. In this paper (p. 601), the data indicate that beef muscle is apparently deficient in cystine and in the following essential amino acids: phenylalanine, tryptophane, methionine, leucine, isoleucine, valine, and possibly threonine. The maximum deficiency is for cystine, followed by cystine +

methionine. Cow's milk shows similar deficiencies in cystine and methionine. These deficiencies are based on chemical or microbiological analyses and some of the apparent deficiencies remain to be tested by animal experimentation.

The biological value of egg protein is not stated in the paper by Mitchell and Block ('46); however, in an earlier paper by Mitchell and Carman ('26) a biological value of 94 was reported when egg protein was fed to young rats at the 8% level. Sumner ('38) found a biological value of 97 for egg when tested with young rats fed a diet in which protein constituted 8%.

Mitchell and Block ('46) cite a biological value of 76 for beef muscle when protein constituted 7.6% of the diet. Hoagland and Snider ('46) reported an average growth-promoting value of 3.15 gm gain per gram of beef protein in 30-day tests with young rats as compared with a gain of 4.28 gm by rats fed dried egg when the diets contained 10% protein.

In view of the apparent deficiency of beef protein in a number of essential amino acids, as compared with egg protein, it seemed desirable to test these apparent deficiencies by appropriate feeding trials with young rats. The purposes of these experiments were to determine (1) whether beef protein was deficient in phenylalanine, tryptophane, methionine, leucine, isoleucine, valine, or cystine, and (2) the effects of the addition of adequate quantities of these amino acids on the growth-promoting properties of the proteins. For comparative purposes, egg protein was tested under similar conditions.

## EXPERIMENTAL

### *Products tested*

Two lots of dehydrated beef were prepared at different times. Lot 1 was prepared from the rounds (rump and shank off) of a steer having a live weight of 910 pounds and a chilled carcass weight of 537 pounds. The carcass graded "low Good." Lot 2 was prepared from the rounds of a steer having a live weight of 872 pounds and a chilled carcass weight of

525 pounds, with the carcass grade of "high Commercial." The rounds were boned out and the lean meat was trimmed free of extraneous fat and connective tissue. The meat was cut into small pieces, frozen at 0°F., ground, and dehydrated at 120°F. to a moisture content of less than 10%. The dehydrated beef was thoroughly extracted with ethyl ether at room temperature. The extracted meat was ground fine and stored in tightly covered tin cans at 25°F. or lower.

Two lots of dried eggs were prepared as follows: fresh eggs were boiled 10 minutes, chilled in cold water, shelled, ground through a meat grinder, and dehydrated at a maximum temperature of 140°F. The dried eggs were thoroughly extracted with ethyl ether, ground, and stored under the same conditions as the dehydrated beef. The dehydrated eggs and beef were analyzed for moisture, ash, fat and nitrogen.

### *Diets fed*

In experiment 1, table 1, the diets were prepared as follows: Dehydrated beef and eggs were incorporated in the diets in such proportions as to supply 10, 12.5, or 15% protein, and certain amino acids were added as indicated. The following quantities of B vitamins were added to 100 gm of diet: thiamine hydrochloride, 0.3 mg; riboflavin, 0.3 mg; pyridoxine hydrochloride, 0.6 mg; calcium pantothenate, 1.5 mg; and choline chloride, 40 mg.

The fat-soluble vitamins were added contained in lard in such amounts that 1 gm of the diet would contain 5 I.U. of vitamin A, 1 unit of vitamin D, and 20 µg of alpha-tocopherol. Sufficient kettle-rendered lard was added to make 10% fat in the diet. Salt mixture amounting to 4% and sufficient dextrin to make 100% completed the diet.

The following amino acids were added to the diets: L-cystine, and the DL forms of each of the following: methionine, phenylalanine, leucine, isoleucine, valine, and tryptophane. In the case of leucine, isoleucine, and valine, only one-half of the nitrogen content of each amino acid was charged against the

nitrogen content of the diet, since only the natural isomers of these amino acids are effective with the rat.

In experiment 2, table 2, cystine was added to diets containing 10% beef protein in the proportions of 0.2, 0.3, 0.4, and 0.5%. For comparative purposes, diets were prepared to contain the same percentages of total nitrogen from beef alone as were present in the corresponding diets containing added cystine.

The feeding tests with rats were conducted in the manner previously described by the writers ('47).

## RESULTS

### *Experiment 1*

In table 1 are summarized the results of feeding tests with young rats to determine the supplemental value of 6 essential amino acids, and of cystine, for the protein in beef when protein constituted 10% of the diet. The supplemental value of cystine for beef protein was tested also at 12.5 and 15% levels of intake. Egg protein was fed at the 10, 12.5, and 15% levels for comparative purposes.

A statistical analysis of the experimental data was made by means of Fisher's "t" values for the significance of differences between the means of the gain in weight per gram of nitrogen consumed. The following probability values were obtained: diet 1 with diets 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16 and 18 — very highly significant ( $< .001$ ); diet 12 with diet 17 — very highly significant ( $< .001$ ); diets 1 and 12 with diet 13 — highly significant ( $< .01$ ); diet 2 with diet 3 — not significant; diet 4 with diet 5 — not significant; diet 3 with diet 16 — not significant; diet 13 with diet 17 — not significant; diet 14 with diet 18 — not significant.

The results of the tests with diets 1 to 5, inclusive, indicate very clearly that beef protein was deficient in either cystine or methionine when protein constituted 10% of the diets. When either amino acid was added to a diet containing 10% beef protein, there was a marked increase in actual gain in

TABLE 1

*Supplemental value of certain amino acids for the protein in dried raw beef in 30-day tests with male albino rats.*

DIET NO. <sup>1</sup>	DESCRIPTION OF DIETS				GAIN IN WEIGHT	FEED CON-SUMED	FEED CON-SUMED FOR 1 GM GAIN IN WEIGHT	GAIN PER GM OF NITRO-GEN CON-SUMED
	Protein	Amino acids added to diets		Nitrogen in diets				
	<i>kind</i>	<i>%</i>	<i>kind</i>	<i>%</i>	<i>%</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>
1	Beef <sup>2</sup>	10.0	...	...	1.60	118	337	2.86
2	Beef	10.0	Cystine	0.20	1.62	146	368	2.52
3	Beef	10.0	Cystine	0.40	1.65	154	370	2.40
4	Beef	10.0	Methionine	0.20	1.62	145	362	2.50
5	Beef	10.0	Methionine	0.40	1.64	149	358	2.40
6	Beef	10.0	Methionine	0.40	1.65	148	358	2.42
			Phenylalanine	0.20				
7	Beef	10.0	Methionine	0.40	1.66	152	365	2.40
			Leucine	0.40				
8	Beef	10.0	Methionine	0.40	1.66	142	342	2.41
			Isoleucine	0.40				
9	Beef	10.0	Methionine	0.40	1.66	143	349	2.44
			Valine	0.40				
10	Beef	10.0	Methionine	0.40	1.65	138	347	2.51
			Tryptophane	0.10				
11	Beef	10.0	Methionine	0.40	1.74	140	338	2.41
			Phenylalanine	0.20				
			Leucine	0.40				
			Isoleucine	0.40				
			Valine	0.40				
			Tryptophane	0.10				
12	Beef	12.5	.....	...	2.00	155	393	2.54
13	Beef	12.5	Cystine	0.40	2.05	163	380	2.33
14	Beef	15.0	...	...	2.40	165	384	2.33
15	Beef	15.0	Cystine	0.40	2.45	160	379	2.37
16	Egg	10.0	.....	...	1.60	149	373	2.50
17	Egg	12.5	.....	...	2.00	158	366	2.32
18	Egg	15.0	.....	...	2.40	164	374	2.28

<sup>1</sup> Each diet, except no. 2, was fed to 8 rats. Diet no. 2 was fed to 7 rats.

<sup>2</sup> Lot 1 of dehydrated beef was used in the experiments reported in table 1.

weight by the rats, and in gain in weight per gram of nitrogen consumed (growth-promoting value). Cystine was just as effective as methionine in supplementing the protein in beef. Although the results in table 1 appear to indicate that 0.4% of either cystine or methionine was slightly more effective than 0.2%, the differences in growth-promoting values were not significant.

The results of the experiments with diets 6 to 10, inclusive, indicate that beef protein was not deficient in either phenylalanine, leucine, isoleucine, valine, or tryptophane when the diets were also supplemented with methionine. The results with diet 11 appear to indicate a depression in growth-promoting value when all the above-named amino acids were added, but this result is due to the increase in nitrogen content of the diet from the addition of 5 amino acids that were not utilized for growth. If the growth-promoting value of diet 11 is calculated on the basis of the nitrogen content of a diet containing 10% protein and 0.4% methionine (diet 5), then the values for the 2 diets are practically the same. The feed consumed per gram gain in weight is practically the same for diets 5 and 11.

When beef protein was increased to 12.5% (diet 12), there was a marked increase in the rate of growth compared to that with the diet containing 10% protein, but the gain in weight per gram of nitrogen consumed was significantly lower. Diet 12 was still somewhat deficient in cystine, since the addition of this amino acid induced a significant increase in growth-promoting value.

When beef protein was increased to 15% (diet 14), there was a further increase in the rate of growth, but a decrease in growth-promoting value. However, the feed consumed per gram gain in weight for this diet was very low as compared with that for most other diets. The addition of cystine to diet 15 did not increase its growth-promoting value. Apparently, 15% beef protein supplied ample cystine and methionine for optimum growth in rats.

The experiments with eggs show that the protein was utilized most efficiently when the diet contained 10% protein, but the actual gain in weight was largest when the protein level in the diet was 15%.

A comparison of the growth-promoting value of the protein in beef with that in egg shows that egg protein was definitely superior to beef protein when the diets contained either 10% or 12.5% of protein, but no supplementary amino acids. However, when beef protein was supplemented with either cystine or methionine, the growth-promoting values of beef protein and egg protein were practically the same. When the diets contained 15% protein but no added cystine, beef protein and egg protein had approximately the same growth-promoting value.

### Experiment 2

In table 2 are summarized the results of experiments to determine the supplemental effects of different proportions of

TABLE 2

*Supplemental value of different proportions of cystine for the protein in dried raw beef in 30-day tests with male albino rats.*

DIET NO. <sup>1</sup>	DESCRIPTION OF DIETS				GAIN IN WEIGHT	FEED CON-SUMED	FEED CON-SUMED FOR 1 GM GAIN IN WEIGHT	GAIN PER GM OF NITROGEN CON-SUMED
	Protein	Amino acids added to diets	Nitrogen in diets					
	kind	%	kind	%	gm	gm	gm	gm
19	Beef <sup>2</sup>	10.00	.	.	1.60	118	349	2.96
20	Beef	10.00	Cystine	0.20	1.62	139	360	2.59
21	Beef	10.13	.	...	1.62	127	366	2.88
22	Beef	10.00	Cystine	0.30	1.63	151	376	2.49
23	Beef	10.19	.	.	1.63	120	349	2.91
24	Beef	10.00	Cystine	0.40	1.65	146	366	2.51
25	Beef	10.31	.	.	1.65	116	338	2.91
26	Beef	10.00	Cystine	0.50	1.66	144	354	2.46
27	Beef	10.38	.	.	1.66	120	344	2.87

<sup>1</sup> Each diet, except no. 20, was fed to 8 rats. Diet no. 20 was fed to 7 rats.

<sup>2</sup> Lot 2 of dehydrated beef was used in the experiments reported in table 2.



cystine on the growth-promoting value of beef protein when the diets contained 10% protein. Statistical tests of the experimental data were made in the same manner as for experiment 1 with the following probability values: diet 19 with diet 20; diet 20 with diet 21; diet 22 with diet 23; diet 24 with diet 25; and diet 26 with diet 27 — all *very highly* significant ( $< .001$ ); diet 20 with diets 22, 24 and 26 — all *not* significant. The results of experiment 2 confirm those of experiment 1, i.e., that the addition of cystine to a diet containing 10% beef protein greatly improved its growth-promoting value. Apparently the addition of 0.2% cystine was nearly, if not quite, sufficient for optimum results since there was no significant increase in growth-promoting value when larger quantities of cystine were added to the diets.

#### DISCUSSION

The experimental data presented in this paper indicate that cystine was as efficient as methionine in supplementing the protein in beef so as to increase its growth-promoting value to that of egg. These results raise the question as to the relative importance of the 2 amino acids in the diet of the young rat. On the basis of the values for cystine and methionine in beef protein and in egg protein given by Mitchell and Block ('46), a diet containing 10% of either protein would contain the following proportions of the 2 amino acids:

*Egg*: methionine, 0.41%; cystine, 0.24%; cystine and methionine, 0.65%. Ratio methionine to cystine, 1.7 : 1. Cystine as per cent of total cystine and methionine, 36%.

*Beef*: methionine, 0.33%; cystine, 0.13%; cystine and methionine, 0.46%. Ratio methionine to cystine, 2.5 : 1. Cystine as per cent of total methionine and cystine, 28.3.

*Beef + 0.2% cystine*: methionine, 0.33%; cystine, 0.33%; cystine and methionine, 0.66%. Ratio methionine to cystine, 1 : 1. Cystine as per cent of total methionine and cystine, 50.

The above data indicate that egg protein, which has a very high biological value at the 8% level, contains methionine and

cystine in the proportion of 1.7 : 1, whereas beef protein with a considerably lower biological value contains these amino acids in the ratio of 2.5 : 1. However, when beef protein was supplemented with 0.2% cystine, the ratio of methionine to cystine was 1 : 1, and the growth-promoting value of the mixture was equal to that of egg protein. The total quantity of cystine and methionine in the egg diet was practically the same as that in the beef + cystine diet. These results indicate that the young rat can utilize methionine and cystine very efficiently for maintenance and growth even when the ratio between the 2 amino acids is 1 : 1. These findings are in contrast to those of Womack and Rose ('41) who conducted experiments with young rats on diets containing pure amino acids as the source of nitrogen. They reported that only a small portion (approximately one-sixth) of the methionine requirement could be satisfied by cystine. The ratio of methionine to cystine under those conditions would be 5 : 1.

Attention is called to the fact that in the experiments herein reported beef protein was found to be deficient only in cystine or methionine, whereas Mitchell and Block ('46) indicate that beef protein is also deficient in phenylalanine, tryptophane, leucine, isoleucine, valine, and possibly threonine. It is possible that beef protein might have been found deficient in amino acids other than cystine or methionine if protein had been fed at a lower level of intake than 10%. Whatever the explanation may be, the results of our experiments with beef protein indicate the importance of checking apparent amino acid deficiencies in proteins, as determined by analysis, by experiments with suitable animals. It is highly important also to determine the level of protein intake at which an amino acid deficiency ceases to exist.

#### SUMMARY

The supplemental values of the amino acids cystine, methionine, phenylalanine, leucine, isoleucine, valine, and tryptophane for beef protein were determined by feeding tests with young male albino rats.

When protein constituted 10% of the diet, beef protein was deficient only in cystine or methionine. When beef protein was supplemented with 0.2% to 0.4% of either cystine or methionine, the growth-promoting value of the protein was equal to that of egg protein.

When the diet contained 12.5% protein, beef protein was somewhat deficient in cystine, but when this amino acid was added, the growth-promoting value was increased to that of egg protein.

When protein constituted 15% of the diet, beef protein was not deficient in cystine and the growth-promoting value was equal to that of egg protein.

The ratio of methionine to cystine in a diet containing 10% egg protein was 1.7 : 1; in a diet containing 10% beef protein the ratio was 2.5 : 1; and in a diet containing 10% beef protein + 0.2% cystine the ratio was 1 : 1.

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# THE ISOLEUCINE REQUIREMENT OF THE INFANT<sup>1</sup>

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## ONE FIGURE

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A survey of the literature indicates that on a body weight basis the nitrogen needs of the infant are 4 to 5 times higher than those of the adult (Albanese, '47). However, the available data do not reveal whether the higher protein requirements of the infant arise from a proportionate increase in all of the amino acids or from a limiting effect created by greater demands of the growing organism for 1 or several of the amino acids. The lack of and the need for this information, which is of obvious importance to practical infant dietetics and the physiology of growth in general, led us to undertake investigations on the amino acid needs of the infant. The determination of the isoleucine requirement of the infant is reported here.

## METHODS AND DIETS

In this as in previous studies (Albanese et al., '47), the requirement of an essential amino acid was estimated from the additional amount needed to restore to physiological levels the nitrogen retention and rate of body weight gain of subjects

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previously maintained on a deficient diet. On the basis of preliminary experiments the following regimen was used: For a week preceding the deficient period the subjects were maintained on a complete diet as constituted by an evaporated milk formula in the first experiment and the deficient diet reinforced with isoleucine in the second experiment. After the deficient diet had been fed for 3 weeks, isoleucine was added in a stepwise manner to a maximum of 8% of the protein intake. Seven-day intervals elapsed between each isoleucine increment tested.

The observations presented here were made on 5 normal healthy male infants who were given the synthetic diets in 5 feedings daily at the rate of approximately 100 cal. per kilogram of body weight and 500 mg of ascorbic acid together with 15 drops of oleum percomorphum daily. The diet periods were of 7 days' duration and consecutive, but collections were omitted on week-ends to avoid complications which might arise from the continued use of restraints. The subjects were immobilized by the use of abdominal restraints which were also designed to hold the urinary adapters in place. Twenty-four-hour urine specimens were collected in bottles containing 1 ml of 15% (by volume) HCl and 1 ml of 10% alcoholic thymol. The feces were collected in 19 cm porcelain evaporating dishes which were held in place by a properly shaped excavation in the mattress, and the daily stools were accumulated under refrigeration for each period in jars containing 200 ml of 70% alcohol. The subjects were weighed daily during the course of the experiment.

The composition of the diets employed is shown in table 1. The diets contained approximately 100 cal. per 100 gm, the percentage caloric distribution being as follows: protein, 14; fat, 36; carbohydrate, 50. The protein moiety of the isoleucine deficient diet was prepared by sulfuric acid hydrolysis of dried beef hemoglobin.<sup>2</sup> This digest which was processed by the procedure previously employed by us in the preparation of human hemoglobin hydrolysates (Albanese and Barnes, '45)

<sup>2</sup> Armour.

was found by analysis to contain 0.60% of isoleucine. The protein equivalent of the final product was estimated as  $N \times 6.25$ . The product was then supplemented with 1.5% of L-tryptophane to replace that which was destroyed during acid hydrolysis. The cystine-poor characteristic of the preparation was improved by the addition of 1% of L-cystine.

TABLE 1

*Composition of diets.*

*(All diets fed at the rate of 100 cal. and 3.5 gm of protein per kilogram body weight.)*

DIETS	A	B	C	D	E	F
	gm	gm	gm	gm	gm	gm
Acid hydrolyzed beef hemoglobin <sup>1</sup>	3.40	3.30	3.30	3.30	3.20	3.10
L-Tryptophane	0.06	0.06	0.06	0.06	0.06	0.06
L-Cystine	0.04	0.04	0.04	0.04	0.04	0.04
L-Isoleucine	0.00	0.02	0.05	0.09	0.18	0.27
Brewers' yeast <sup>2</sup>	1.00	1.00	1.00	1.00	1.00	1.00
Olive oil	4.00	4.00	4.00	4.00	4.00	4.00
Dextri-maltose #2	9.60	9.60	9.60	9.60	9.60	9.60
Arrowroot starch	2.30	2.30	2.30	2.30	2.30	2.30
Salt mixture <sup>3</sup>	1.60	1.60	1.60	1.60	1.60	1.60
Water	78.00	78.00	78.00	78.00	78.00	78.00
Total	100.00	100.00	100.00	100.00	100.00	100.00
Estimated isoleucine content (mg)	36	60	90	126	216	306

<sup>1</sup>  $N \times 6.25 =$  gm of protein containing 0.60% of isoleucine by chemical analysis.

<sup>2</sup> Kindly supplied by the Mead Johnson and Company and found to contain 3.0% isoleucine by chemical analysis.

<sup>3</sup> The salt mixture employed had the following composition (measured in gm):  $\text{FeSO}_4$  0.9,  $\text{NaCl}$  6,  $\text{Ca gluconate}$  48,  $\text{Ca(OH)}_2$  12,  $\text{KH}_2\text{PO}_4$  7,  $\text{KCl}$  6, and  $\text{MgO}$  0.1.

The isoleucine supplement was added to the diet in the step-wise fashion (table 1). Owing to uncertainties regarding the complete human requirements of B complex vitamins, brewers' yeast was employed instead of a mixture of the synthetically available vitamins. The quantities of isoleucine derived from this source appear to be approximately 16 mg per gram

(Carter and Phillips, '44). Thus, the amount of isoleucine per kg of infant body weight provided by the diets can be estimated roughly (table 1). The final nitrogen content of each batch of diet was determined by micro-Kjeldahl analysis.

The evaporated milk formula employed for the precontrol period in the first experiment had the following composition: evaporated milk, 40 ml (55.3 cal.) ; corn syrup, 6 ml (17.7 cal.) ; water, 54 ml. This supplied approximately 73 cal. per 100 ml and the nitrogen content of each batch was controlled by micro-Kjeldahl analysis.

The data on nitrogen retention were calculated from the results of nitrogen determinations of the 24-hour urine collections, analyses of the pooled feces for each period, and from computation of the daily nitrogen intake based on food consumption records and the known nitrogen content of the diets.

Blood samples (10 ml) were collected over lithium oxalate by vena puncture on the last day of each diet period. The hemoglobin concentration of these specimens was determined colorimetrically in the Klett-Summerson photoelectric colorimeter. The total plasma proteins, albumin, globulin and non-protein-nitrogen were determined by the procedure recently described by us (Albanese, Irby and Saur, '46).

#### RESULTS AND DISCUSSION

The data obtained from the first experiment of this study (table 2) show that the omission of isoleucine from the diet caused an immediate and sustained drop of the daily weight gain and nitrogen retention below the control levels of all the subjects. Since the growing organism is normally in a state of high positive N balance, a drop from the nitrogen retention values characteristic of the individual must be given the same interpretation as the establishment of a negative N balance in the adult, namely, that isoleucine is a dietary essential for the infant. This inference is also corroborated by the observed decrements in rate of weight gain. Following the deficient period, all 3 subjects were given diet D which provided ap-

proximately one-third of the isoleucine calculated for the evaporated milk formula (Williamson, '44). It will be noted that this diet restored the N retention and weight coefficients to the levels attained with the evaporated milk diet. Since the feeding of diets E and F failed to augment the magnitude of these criteria, it could be concluded that the 126 mg of L-isoleucine per kg of body weight supplied by diet D are adequate (if not more than adequate) for infants of this age.

TABLE 2

*Effect of dietary isoleucine on the nitrogen retention and body weight of the infant.*  
(All results given as daily averages.)

INITIAL AGE AND WEIGHT OF SUBJECT	DIET	EVAPO- RATED MILK						
			A	A	A	D	E	F
	Isoleucine intake, mg/kg/day	306	36	36	36	126	216	306
H. G., male, 4 mos. 4.735 kg	N intake (gm)	2.85	2.69	2.90	2.80	2.77	3.10	3.05
	N retention (mg/kg)	220	81	65	43	246	248	224
	Weight change (gm)	+31	-21	+7	-4	+28	+36	+23
W. T., male, 10 mos. 6.965 kg	N intake (gm)	4.10	4.01	3.83	4.04	4.29	4.13	4.23
	N retention (mg/kg)	180	63	92	64	192	164	174
	Weight change (gm)	+30	+21	+4	+9	+47	+42	+30
A. H., male, 9 mos. 6.173 kg	N intake (gm)	3.30	3.21	2.99	3.20	3.08	3.26	...
	N retention (mg/kg)	200	92	71	59	291	212	...
	Weight change (gm)	+32	-28	0	-28	+28	+28	...

Moreover, the fact that diets D, E, and F sustained N retention levels and weight gains comparable to those found with the evaporated milk formula further suggests that the nitrogen moiety of these diets, namely, the beef hemoglobin hydrolysate as modified for this study, has a biological value similar to that of the casein and lactalbumin mixture found in evaporated milk.

In order to ascertain the isoleucine requirement of the infant more closely than was permitted by our first experiment,



2 more subjects were similarly studied with the exception that diets B and C, which supplied, respectively, 60 and 90 mg of isoleucine per kg, were fed after 2 weeks of the deficient regimen. The results of this experiment (fig. 1) indicate that the N-retention and daily body weight change were restored

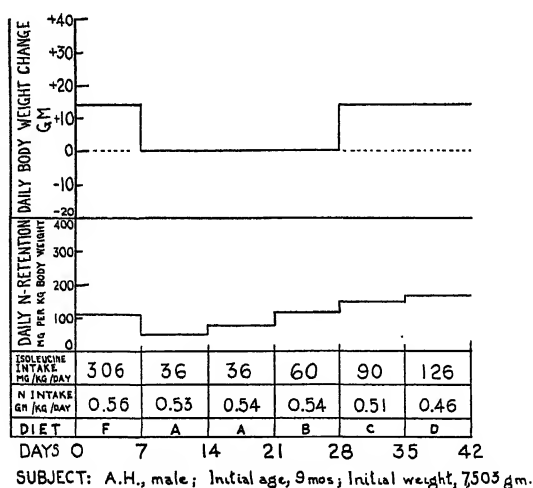
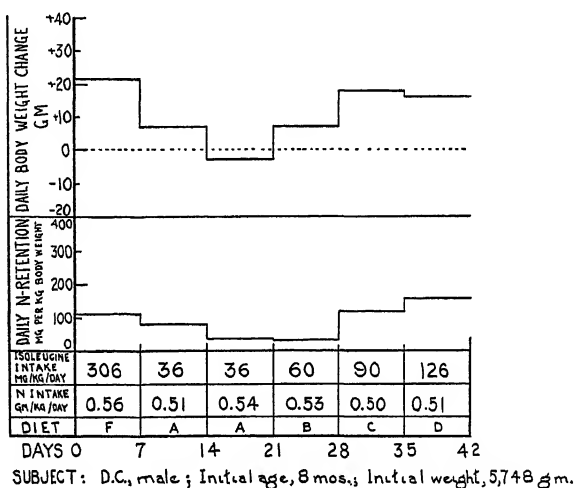


Fig. 1 Effect of variations of isoleucine content of the diet on the nitrogen retention and body weight changes of the infant.

to the control levels by diet C but not by diet B and signify therefore that infants of this age group require approximately 90 mg of L-isoleucine per kilogram per day. This value is approximately one-third of that which is normally supplied by milk formulae fed at comparable nitrogen and caloric levels.

Measurements of the blood proteins during the experiments disclosed that, unlike the tryptophane deficient diet, the isoleucine deficient diet had no appreciable effect on the blood plasma protein and hemoglobin levels of the infant. This finding is of interest in view of the low isoleucine content of these tissue proteins.

Careful clinical examinations of the infants throughout the course of these experiments disclosed that the feeding of an isoleucine poor diet for periods of 2-3 weeks does not induce any detectable clinical symptoms in the infant.

#### SUMMARY

On the basis of changes in rate of growth and nitrogen retention induced by the fractionally supplemented control diets following 3 weeks of the isoleucine deficient regimen, it has been estimated that the infant requires approximately 90 mg of L-isoleucine per kilogram per day.

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# THE ROLE OF VITAMIN D IN THE UTILIZATION OF PHYTIN PHOSPHORUS<sup>1</sup>

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## INTRODUCTION

Earlier studies (Spitzer and Phillips, '45a, '45b) demonstrated that the rat could efficiently utilize the phytin phosphorus of soybean oil meal when adequate vitamin D and a favorable Ca-P ratio were employed. These studies indicated that the phytin phosphorus was made available by the action of an intestinal enzyme, phytase, capable of splitting the phytin molecule. In later studies, Boutwell et al. ('46) also suggested the importance of an intestinal phytase in the utilization of phytin phosphorus. These same investigators also showed that the availability of phytin phosphorus was greatly enhanced when vitamin D was added to the ration. Other evidence in the literature has shown the importance of vitamin D in the utilization of phytin phosphorus. Krieger and co-workers ('40, '41) demonstrated that in the absence of vitamin D, phytic acid phosphorus was not available to the rat, but when vitamin D was fed, more of the phosphorus was utilized. In studies with chicks, McGinnis and co-workers ('44) showed that phytin phosphorus was poorly available in

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the absence of vitamin D, but that it was nearly as available as inorganic phosphorus when adequate vitamin D was fed. Singsen and Mitchell ('45) also reported that vitamin D increased the utilization of phytin phosphorus in chicks.

Thus it seems apparent that the availability of phytin phosphorus is dependent upon the action of an intestinal phytase and upon an adequate level of vitamin D in the ration. It occurred to us that vitamin D might be necessary either for phytase formation or for its proper functioning. It seemed possible that if vitamin D was necessary for phytase formation, tissues from rats receiving varying amounts of vitamin D would vary in their phytase content. The following experiments were designed to obtain information about this possible relationship. Phytase activity was measured in rats fed calcium phytate and inorganic phosphorus and varying amounts of vitamin D.

#### EXPERIMENTAL

Weanling male rats of Sprague-Dawley breeding were divided in 4 lots and fed rations outlined in table 1. The basal ration (lot I) contained 0.26% inorganic phosphorus and was devoid of vitamin D. Lot II received the same ration but in addition was given 100 A.O.A.C. units of calciferol by dropper 3 times each week. Lots III and IV differed from the first 2 lots in that the phosphorus was furnished as calcium phytate.<sup>3</sup> Both of these rations contained 0.23% calcium phytate phosphorus and 0.02% inorganic phosphorus. Lot IV received 100 A.O.A.C. units of calciferol by dropper 3 times weekly.

The phosphorus level in all rations was kept as near as possible to 0.25%, a level slightly below the optimum phosphorus intake for weanling rats (Spitzer and Phillips, '45b). It was felt that this level would afford a more critical measure of the availability of calcium phytate and the effect

<sup>3</sup> Phytin is the calcium-magnesium salt of phytic acid. Phytic acid is the hexaphosphoric acid ester of inositol. These compounds and other salts of phytic acid are probably rendered available by similar mechanisms.

of vitamin D on this process. The calcium content of all rations was kept as near as possible to 0.6%.

Rats were decapitated on the thirty-second day of the experiment. Approximately a 1-gm sample of small intestine was removed, weighed and frozen. In all cases the sample was taken by severing the small intestine at the pylorus and using the duodenum for analysis. The femora were also

TABLE 1  
*Composition of rations (per 100 gm ration).*

BASAL MIXTURE	LOT I	LOT II	LOT III	LOT IV	ALL LOTS:	
					(Vitamins/100 gm basal mixture)	
	<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>		<i>mg</i>
Blood fibrin	14.0	14.0	14.0	14.0	B-carotene	2.0
Sucrose	77.75	77.75	78.15	78.15	Riboflavin	0.3
Corn oil	4.0	4.0	4.0	4.0	Thiamine HCl	0.3
Low Ca-P salts <sup>1</sup>	2.0	2.0	2.0	2.0	Pyridoxine	0.3
CaHPO <sub>4</sub>	1.37	1.37	...	...	Ca pantothenate	2.5
CaCO <sub>3</sub>	0.48	0.48	...	...	Niacin	2.5
Ca phytate	...	...	1.45	1.45	Vitamin D <sup>2</sup>	
Inositol	0.1	0.1	0.1	0.1		
Choline chloride	0.3	0.3	0.3	0.3		
<i>Phosphorus <sup>3</sup> in %:</i>						
Inorganic P	0.26	0.26	0.02	0.02		
Phytin P						
(Ca phytase)	...	...	0.23	0.23		
Total P	0.26	0.26	0.25	0.25		

<sup>1</sup> Low Ca-P salt mixture used earlier by Spitzer and Phillips ('45a).

<sup>2</sup> Rats in lots II and IV were given 100 A.O.A.C. units of calciferol by dropper 3 times each week.

<sup>3</sup> Inorganic P analyses were made, using the method of Fiske and Subbarow ('25). Phytin P analyses were made, using the method of McCance and Widdowsen ('35).

removed and bone ash values determined as previously described (Spitzer and Phillips, '45a). All rats were starved for 36 hours prior to the time they were sacrificed in order to clear the intestine of food materials. Samples of the small intestine were later removed from the refrigerator and phytase determinations made.

## RESULTS

*Growth and bone ash studies*

Growth and bone ash data are summarized in table 2. Rats receiving the inorganic phosphorus (lots I and II) made more rapid gains than did those receiving the calcium phytate phosphorus (lots III and IV). Rats given vitamin D (lots II and IV) made somewhat poorer gains than did those on the basal ration (lots I and III).

Under the conditions of this experiment, vitamin D supplementation had little or no effect on bone ash values when inorganic phosphorus was fed. This contrasts sharply with the effect on the utilization of calcium phytate phosphorus.

TABLE 2

*Effect of vitamin D on growth and bone ash in rats fed inorganic and calcium phytate phosphorus.*

LOT NUMBER	NO. OF RATS	PHOSPHORUS Form and amount in ration	AVERAGE BODY WEIGHT AT 32 DAYS FROM IN- ITIAL WEIGHT OF 40	BONE ASH
			gm	
I	8	Inorganic (0.26%)	122	47.1
II	8	Inorganic (0.26%) + vitamin D	114	48.0
III	8	Calcium phytate (0.25%)	80	23.3
IV	8	Calcium phytate (0.25%) + vitamin D	69	43.4

Supplementation of vitamin D when calcium phytate was fed had a marked effect on the utilization of this organically bound form of phosphorus (lots III and IV). The bone ash values of the rats in lot III averaged 23.3%. These same animals showed marked signs of rickets throughout the experiment. Supplementation with vitamin D (lot IV) markedly increased the utilization of the calcium phytate phosphorus. The bones of these rats contained 43.4% ash and no signs of rickets were observed throughout the study. Considering the slightly lower phosphorus level in the calcium phytate ration (lot IV) as compared to that for the inorganic control (lot II), it appears that the calcium phytate phos-

phorus was nearly as efficiently utilized as was the inorganic phosphorus when adequate vitamin D was fed.

During the course of the experiment a very marked alopecia developed in lot IV. No marked signs of alopecia were observed in the other lots. Lot III rats were especially well-coated.

### *Development of a phytase method*

The phytase method outlined by Spitzer and Phillips ('45b) was adapted for quantitative determination. Frozen tissue of known weight, usually 1 gm, was homogenized by the Potter-Elvehjem technique ('36). The sample was made to volume (25 ml) with distilled water. A suitable buffer and the substrate (sodium phytate) contained in test tubes were diluted to a 9 ml volume and brought to the incubation temperature. To one tube was added 1 ml of the tissue homogenate and to the control tube, 1 ml of distilled water. Using this procedure it was possible to check specifically any phytase activity by measuring the increase in inorganic phosphorus resulting from hydrolysis of the sodium phytate. The medium was mixed thoroughly and incubated at 37°C. Samples were removed at intervals, added to an equal volume of 10.0% trichloroacetic acid and then analyzed for liberated phosphorus according to the method of Fiske and Subbarow ('25). The assays were carried out in dimmed and artificial light in an effort to minimize the possibility of contamination by vitamin D. The increase in inorganic phosphorus is a measure of the phytase activity.

*Effect of buffers.* A 0.05 M diethylamine citrate buffer inhibited phytase activity. A similar concentration of sodium borate proved to be a satisfactory buffer, but the one which allowed greatest phytase activity was a 0.05 M sodium barbitol. Maximum activity was observed at a slightly alkaline pH. Experiments reported herein were conducted at pH 8 using a 0.05 M sodium barbitol buffer.

*Effect of time.* Table 3 shows the effect of time on phytase activity. Inorganic phosphorus was liberated very rapidly



during the first few minutes of assay. A slower, steadier rate of liberation was then noted.

*Phytase determinations on tissue samples.* Results showing the effect of vitamin D on phytase activity in rats fed the experimental rations are summarized in table 3. Phytase analyses were made on samples from 8 rats in each lot. Each listed value represents an average of from 2 to 12 assays.

Tissues from rats fed the different sources of phosphorus and various amounts of vitamin D showed no marked variations in phytase activity. When a 5-minute assay period was

TABLE 3

*The effect of vitamin D on phytase activity in rats fed inorganic and calcium phytate phosphorus.*

LOT NUMBER	PHOSPHORUS Form in ration	MICROGRAMS INORGANIC PHOSPHORUS LIBERATED PER GRAM OF TISSUE HOMOGENATE <sup>1</sup>						
		1 min.	3 min.	5 min.	15 min.	30 min.	60 min.	120 min
I	Inorganic	....	....	2,500	3,000	....	5,900	8,400
II	Inorganic + vitamin D	....	....	2,300	3,100	....	5,500	7,600
III	Calcium phytate	1,900	2,000	2,500	3,200	3,800	5,700	9,700
IV	Calcium phytate + vitamin D	1,500	1,900	2,400	3,300	4,300	5,900	7,700

<sup>1</sup> Each value listed is an average of from 2 to 12 determinations. Eight animals were used in each lot. No determinations were made at 1 min., 5 min., or 30 min. for lots I and II.

used, an average of 2,300 to 2,500  $\mu\text{g}$  of inorganic phosphorus per gram of tissue was liberated by all samples. At 60 minutes, approximately equal amounts (5,500–5,900  $\mu\text{g}$  per gram of tissue) of phosphorus had been freed by all samples. Small variance was observed between samples from different lots when other assay times were used. However, since there was also some variance in samples from the same lot, no definite difference could be noted. In general, as is indicated in table 3, all samples showed approximately the same phytase activity.

*Vitamin D in vitro.* Results of a limited number of assays indicated that the addition of vitamin D to tissue homogenates in vitro did not affect phytase activity.

#### DISCUSSION

The importance of vitamin D in the utilization of organically bound phosphorus is demonstrated. Although calcium phytate phosphorus was poorly available in the absence of vitamin D, it was readily available when adequate D was supplied. These results confirm the findings of earlier investigations by Spitzer and Phillips ('45b), Boutwell et al. ('45), Krieger and co-workers ('40, '41), and others.

In the light of these results, the apparent contradictions of earlier studies (Boutwell et al., '46; Spitzer and Phillips, '45a, '45b) on the availability of phytin phosphorus might be explained on the basis of the vitamin D content of the rations used.

The present studies indicate that although vitamin D is necessary for the utilization of calcium phytate phosphorus, this vitamin is not necessary for the formation of the enzyme phytase which makes possible the splitting of the phytin molecule in the intestine. The small intestines of rats fed inorganic or bound phosphorus with or without vitamin D, all showed equal phytase activity as measured by the phytase assay outlined above. These results indicate that vitamin D apparently does not function by aiding phytase formation.

It is interesting to note that although vitamin D increased the utilization of the calcium phytate phosphorus (table 2), growth was somewhat poorer when this vitamin was fed (lot IV). Rats receiving phytate and vitamin D (lot IV) also showed a marked hair loss which did not occur when only calcium phytate was fed (lot III). The pattern of the hair loss was similar to that of an inositol deficiency described by Cunha et al. ('43) and Spitzer and Phillips ('46). In these studies in which inositol deficiencies were reported, the rations contained considerable phytin. If in the presence of adequate vitamin D phytin phosphorus was readily utilized, and if a

molecule of phytin was completely hydrolyzed, the final products would be 1 inositol and 6 phosphoric acid molecules. Theoretically, this inositol should be available to the rat. Yet, an inositol deficiency developed in our experimental animals even though the ration contained 0.1% inositol in addition to that resulting from the hydrolysis of the calcium phytate. It is possible that the slightly imperfect utilization resulted in an antagonistic intermediate which increased the requirement of inositol.

#### SUMMARY

The effect of vitamin D on the utilization of calcium phytate phosphorus and on inorganic phosphorus has been studied in the rat.

The addition of vitamin D to a ration containing nearly optimum inorganic phosphorus had little or no effect on phosphorus utilization as indicated by bone ash studies. However, the addition of vitamin D to a ration containing calcium phytate greatly enhanced phosphorus utilization. Therefore, in the presence of adequate vitamin D, bone ash values indicated that the calcium phytate phosphorus was almost as effectively utilized as the inorganic phosphorus.

The phytase content of the small intestines of rats fed calcium phytate and varying amounts of vitamin D showed no difference in phytase activity. Apparently vitamin D was not necessary for phytase formation. It is suggested that vitamin D functions in a secondary role in the utilization of phytin phosphorus.

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# THE UTILIZATION BY THE CHICK OF PHOSPHORUS FROM DIFFERENT SOURCES<sup>1</sup>

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Recent changes in animal feeding practices have increased the need for inorganic phosphorus supplements to supply part of the phosphorus formerly obtained from animal protein supplements and steamed bone meal. This has led to a greater production and use of thermally defluorinated superphosphate and phosphate rock. The desirability of defluorinated phosphate products as phosphorus supplements is indicated by the results of a number of workers (Fraser, Hoppe, Sullivan and Smith, '43; McConnell, Insko and Buckner, '44; Barrentine, Maynard and Loosli, '44, and others). Considerable variability has been found to exist, however, among products produced under different conditions. This is particularly true of the superphosphates (Bird et al., '45).

Differences in availability are attributable in large part to variation in the content of pyrophosphate and metaphosphate. In a series of collaborative studies, Ellis et al. ('45) and Bird et al. ('45) have shown that the calcium pyrophosphates and calcium metaphosphates which are likely to occur in defluorinated phosphate products are less available than the ortho form of phosphate. These 3 forms of phosphorus are capable of existing singly or together in the end product following

<sup>1</sup> This work was supported in part by the establishment at Cornell University of a fellowship by the International Minerals and Chemical Corporation, Chicago, Illinois.

the defluorinating process. Furthermore, each of these calcium phosphates, ortho, pyro, and meta, is capable of existing in a number of different enantiotropic crystalline forms and, in the case of the calcium metaphosphate, a vitreous form is also possible. The temperature at which the fluorine is eliminated is a primary factor in determining the type of phosphorus compound formed; however, other factors such as rate of cooling may influence the final crystalline modification or result in a vitreous system.

More exact information would be valuable on the relative biological availability of each of these compounds which may be expected to occur in defluorinated phosphates. This is true not only from the standpoint of determining their individual feeding values, but also in evaluating the over-all availability of the defluorinated phosphate material in which several forms may occur, depending upon the treatment given. At the same time more information is needed concerning the relative value of the commonly-used phosphorus supplements. Accordingly, a series of experiments has been conducted to determine the relative availability of a number of pure phosphate compounds and other common phosphorus supplements when used as sources of phosphorus in a low phosphorus basal diet. The importance of crystalline structure and solubility in determining availability has been investigated also. The results of these studies are reported in this paper.

#### EXPERIMENTAL DIET AND MATERIALS

Although studies have been made of the relative efficiency of different phosphorus supplements for the chick, most of these experiments are open to possible criticism because the basal diets used have been too high in phosphorus to permit the most efficient study of single supplements. This has been true particularly in the case of studies involving the pyrophosphates and metaphosphates. In many instances, the test supplements have raised the phosphorus level of the diet considerably above the requirement of the chick, whereas it would seem desirable to make comparative studies near the critical

level of phosphorus required. With these facts in mind, a basal diet has been developed which does not contribute a significant percentage of the phosphorus requirement of the chick. In formulating a suitable basal diet low in this element, the principal difficulties encountered are the organic phosphorus in the cereal grains and the phosphorus associated with

TABLE 1  
*Low phosphorus basal diet.*

	%		%
Corn starch	58.5	Vitamin mixture	0.5
Dried blood fibrin	25.0	(see below)	
Gelatin	5.0	Salt mixture	6.0
Ground cellophane	3.0	(see below)	
Soybean oil	2.0		

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SALT MIXTURE	%	VITAMINS	gm/100 lbs.
NaCl	0.6	Thiamine	0.15
KCl	0.5 <sup>1</sup>	Riboflavin	0.225
MgSO <sub>4</sub> ·7 H <sub>2</sub> O	0.4	Ca pantothenate	0.50
FeSO <sub>4</sub> ·7 H <sub>2</sub> O	0.06	Pyridoxine	0.225
MnSO <sub>4</sub> ·4 H <sub>2</sub> O	0.03	p-aminobenzoic acid	5.00
ZnCl <sub>2</sub>	0.001	Niacinamide	0.80
CoCl <sub>2</sub> ·6 H <sub>2</sub> O	0.0003	Inositol	50.00
KI	0.0033	Choline	100.00
CuSO <sub>4</sub> ·5 H <sub>2</sub> O	0.0017	Tocopherols in oil	70.00
Ca + P supplements <sup>2</sup>		Vitamin K	0.10
		Folic acid	0.15
		Biotin	10 mg
		Vitamin A alcohol	50 mg
		Vitamin D <sub>3</sub>	22,700 AOAC
			chick units

<sup>1</sup> When KH<sub>2</sub>PO<sub>4</sub> was used as a source of phosphorus, no KCl was added.

<sup>2</sup> Variable, depending on the supplements used. When necessary, adjustments were made in level of corn starch.

most of the common protein supplements used in chick diets. Following the suggestion of Jones ('38), dried blood fibrin was found to be low in phosphorus as well as an excellent source of protein. The cereal grains were eliminated by using corn starch. The composition of the basal diet used in all the studies reported in this paper is given in table 1. It contains



approximately 0.03% phosphorus or less than one-tenth the amount required by the chick.

The supplements used in these experiments together with their analytical and solubility data are listed in table 2. They include the commonly-used supplements — steamed bone meal, defluorinated superphosphate (defluorination temperature

TABLE 2  
*Analytical and solubility data for phosphorus supplements.*

MATERIAL	Ca	P	% TOTAL P SOLUBLE IN 0.4% HCl <sup>1</sup>
	%	%	
KH <sub>2</sub> PO <sub>4</sub>	...	22.7	100.0
Beta-tricalcium phosphate	38.5	19.7	84.6
Tricalcium phosphate <sup>2</sup>	36.9	19.0	90.5
Dicalcium phosphate <sup>2</sup>	28.9	22.7	100.0
Monocalcium phosphate <sup>2</sup>	15.3	24.8	100.0
Steamed bone meal	31.8	14.2	97.6
Defluorinated superphosphate	36.1	15.5	86.8
Calcined rock phosphate	32.4	18.6	87.5
Fused rock phosphate	29.3	12.0	89.0
Vitreous calcium metaphosphate	17.6	25.7	54.2
Vitreous sodium metaphosphate	...	29.5	100.0
Potassium metaphosphate	..	26.6	93.5
Beta-calcium metaphosphate	21.3	30.2	3.2
Gamma-calcium metaphosphate	21.3	29.8	5.7
Calcium acid pyrophosphate, crystalline	19.0	28.5	98.6
Alpha-calcium pyrophosphate	31.0	24.0	69.8
Beta-calcium pyrophosphate	31.3	24.1	54.2
Gamma-calcium pyrophosphate	31.3	24.2	73.4
Calcium phytate	12.9	12.6	13.7

<sup>1</sup> Method of Reynolds et al. ('44): 1 gm material digested in 100 ml 0.4% HCl for 1 hour.

<sup>2</sup> Reagent grade material.

1150°C.), calcined rock phosphate, fused rock phosphate, and reagent grades of orthophosphates, mono-, di-, and tricalcium phosphate. Calcined rock phosphate is defluorinated at temperatures below that required for complete melting, and fused rock phosphate freed from its fluorine at temperatures above that needed for complete melting. In addition, a number of pure pyrophosphates, metaphosphates, and pure *beta* tri-

calcium phosphate were included. A crude calcium phytate was used since an important part of the phosphorus in many rations consists of phytic acid and its salts.

The sample of *alpha* calcium pyrophosphate was produced by a commercial source for use in these experiments. All of the other pure pyrophosphates and metaphosphates as well as *beta* tricalcium phosphate were obtained from the U.S.D.A. Agricultural Research Administration, Beltsville, Maryland. A detailed description of these products, together with methods of preparation, has been given by Hill et al. ('44, '45 and '48). It is sufficient to point out that the different Greek letter-designated modifications of calcium metaphosphate [ $\text{Ca}(\text{PO}_3)_2$ ] and calcium pyrophosphate [ $\text{Ca}_2\text{P}_2\text{O}_7$ ] differ among themselves only in crystal structure and not in chemical composition. The designations begin with the *alpha* form which is stable at the melting point. The *gamma* calcium metaphosphate was only about 80% pure, the impurity being mainly  $\text{Ca}_2\text{HP}_3\text{O}_{10}$ . The *beta* tricalcium phosphate is that form of pure  $\text{Ca}_3(\text{PO}_4)_2$  which is stable at ordinary temperatures and which predominates in defluorinated superphosphate and calcined rock phosphate. The reagent grades of mono-, di-, and tricalcium phosphate were obtained from commercial sources, and, as Hodge, LeFevre and Bale ('38) have pointed out, they are seldom pure compounds corresponding to the theoretical composition. The alkaline salts, vitreous sodium metaphosphate and potassium metaphosphate, were studied although they are not expected to occur in thermally defluorinated phosphates.

#### PROCEDURE

White Leghorn cockerels were used as experimental animals. Lots of 15 1-day-old chicks were placed on the experimental diets in electrically heated brooders with food and water supplied ad libitum. All chicks were weighed individually at weekly intervals during the 4-week experimental period. At the end of the fourth week, 10 representative chicks in each lot were killed and the left tibia removed for bone ash

determination. In lots with 10 chicks or less surviving the 4-week experimental period, all survivors were used for bone ash determinations.

Each phosphorus supplement was added to the basal diet at 2 levels calculated to raise the phosphorus content to 0.4% and 0.8%, respectively. The 0.4% level is near the minimum requirement of the chick for phosphorus. It was thought that by supplying phosphorus at this level, a more critical comparison of relative availability would be possible, particularly with the more available phosphates. By increasing the level of phosphorus to 0.8%, any degree of availability in the more unavailable materials should become apparent. Also, it was thought that if a compound exhibited any toxic effects, results should be poorer at the higher level. Where the Ca:P ratio of the supplement added was less than 2:1, this ratio was established by the addition of pure calcium carbonate. In the case of bone meal, fused rock phosphate, and the defluorinated superphosphate used, the ratio was unavoidably greater than 2:1, being 2.18:1 for bone meal, 2.43:1 for fused rock phosphate, and 2.25:1 for superphosphate. The results presented here were collected from 4 different experiments. Since all studies could not be conducted simultaneously, lots receiving  $\text{KH}_2\text{PO}_4$  were used as positive controls in each experiment to insure that the experiments were comparable. This phosphate was selected for its known ready availability since it is completely water soluble, and it gave uniformly good results in preliminary studies.

#### RESULTS AND DISCUSSION

The results obtained on growth, bone ash, and mortality are presented in table 3. When the basal diet was fed without additional phosphorus, the chicks died within a short period. Since there is no report in the literature describing the effects of severe phosphorus deficiency in young chicks, a brief description is appropriate here. The effects of the deficiency are acute, 100% mortality occurring in a few days. The chicks eat well for 3 or 4 days and make small initial gains in

weight. After this, however, there is a rapid decline in appetite. Coincident with the loss of appetite, a general weakness develops and the chicks are reluctant to stand or use their legs. The first mortality usually occurs by the fifth or sixth day and all the chicks are dead by the tenth to twelfth day. During the last stages of the deficiency, the chicks usually lie on their

TABLE 3

*The relative availability of different phosphorus supplements for the chick.*

SUPPLEMENT	0.4% PHOSPHORUS			0.8% PHOSPHORUS		
	Wt. 4th wk.	Bone ash	Mor- tality	Wt. 4th wk.	Bone ash	Mor- tality
	<i>gm</i>	<i>%</i>	<i>%</i>	<i>gm</i>	<i>%</i>	<i>%</i>
None <sup>1</sup>	...	...	100.0	...	...	...
Potassium phosphate, monobasic	296	46.5	4.4	291	45.6	0.0
Beta-tricalcium phosphate	281	44.2	0.0	299	46.3	0.0
Tricalcium phosphate (reagent)	272	44.8	0.0	307	48.2	0.0
Dicalcium phosphate (reagent)	303	43.9	6.7	300	47.2	0.0
Monocalcium phosphate monohydrate	307	45.9	13.4	282	47.2	0.0
Steamed bone meal	250	43.5	0.0	290	48.5	0.0
Fused rock phosphate	241	40.7	13.4	265	47.1	0.0
Defluorinated superphosphate	211	38.7	6.7	279	48.0	0.0
Calcined rock phosphate	211	40.0	6.7	271	47.4	0.0
Calcium acid pyrophosphate	285	41.6	0.0	273	43.5	6.7
Alpha-calcium pyrophosphate	...	...	100.0	...	...	100.0
Beta-calcium pyrophosphate	...	...	100.0	...	...	100.0
Gamma-calcium pyrophosphate	...	...	100.0	...	...	100.0
Vitreous calcium metaphosphate	186	30.9	66.7	247	38.4	33.3
Vitreous sodium metaphosphate	155	32.2	80.0	128	35.3	73.2
Potassium metaphosphate	...	...	100.0	...	...	100.0
Beta-calcium metaphosphate	...	...	100.0	...	...	100.0
Gamma-calcium metaphosphate	...	...	100.0	...	...	100.0
Calcium phytate	...	...	100.0	...	...	100.0

<sup>1</sup> Phosphorus level of diet 0.03%.

sides. These results show that the young chick requires an immediate source of available phosphorus for the maintenance of vital functions. The results are approximately the same whether or not calcium is added to the basal diet.

It is evident from table 3 that all the pure or relatively pure orthophosphates studied were excellent sources of phos-

phorus for the chick when added to the basal diet as the sole phosphorus supplement.  $\text{KH}_2\text{PO}_4$ , pure *beta* tricalcium phosphate, and the reagent grades of mono-, di-, and tricalcium phosphate all permitted growth of from 270 to 307 gm at 4 weeks, and bone ash values of from 43.9% to 46.5%, with very low mortality at the 0.4% phosphorus level. At the 0.8% level, growth ranged from 282 gm to 307 gm, and bone ash values from 45.8% to 48.2%.

Steamed bone meal was slightly less available than the first 5 salts, but was a very good source of phosphorus. The results given for bone meal are an average of 2 samples which varied considerably. The results of these experiments indicate that in some instances bone meal may be less available than is generally thought. When it supplied 0.8% phosphorus, bone meal was as good as pure *beta* tricalcium phosphate.

The results obtained with fused rock phosphate, defluorinated superphosphate and calcined rock phosphate demonstrated the value of these materials as phosphorus supplements. However, they were less available than potassium phosphate or the various calcium orthophosphate salts. They were also less available than bone meal. These 3 products were of equal value when they were used to raise the phosphorus content of the diet to 0.8%. At this level they were only slightly less satisfactory than steamed bone meal or pure orthophosphate. The calcined rock phosphate and defluorinated superphosphate were predominantly *beta* tricalcium phosphate as shown by x-ray analyses, and the fused rock was mostly *alpha* tricalcium phosphate. Apparently, therefore, the crystalline structure has little to do with availability in the case of these compounds.

Of the 4 pyrophosphates studied, only the crystalline calcium acid pyrophosphate was available. This compound was a good source of phosphorus, particularly at the marginal level. At the higher level, the results were less favorable than would be expected on the basis of results at the critical level, growth being actually less and bone ash values only slightly better. It seems probable either that the op-

timum level of phosphorus from this compound is less than 0.8%, or that for best results it should be supplemented with other sources of phosphorus. The availability of this product probably derives from the fact that it is easily hydrated in the body to monocalcium phosphate as follows:  $\text{CaH}_2\text{P}_2\text{O}_7 \xrightarrow{\text{H}_2\text{O}} \text{Ca}(\text{H}_2\text{PO}_4)_2$ . The *alpha*, *beta*, and *gamma* calcium pyrophosphates were completely unavailable or very nearly so. This occurred in spite of the fact that they are appreciably soluble in 0.4% HCl. There was no significant difference in the survival time of chicks receiving these supplements and those receiving the unsupplemented basal diet, even when a sufficient quantity of these compounds was used to raise the phosphorus content of the diet to 0.8%. These compounds did not appear to be toxic, however. It is evident that here again the different crystalline forms did not vary in their availability.

Of the 5 metaphosphates studied, only the vitreous calcium metaphosphate and the vitreous sodium metaphosphate exhibited any availability. The 2 crystalline forms of calcium metaphosphate were identical in their lack of availability. Although the vitreous calcium metaphosphate is appreciably soluble in dilute HCl, and has been reported to be somewhat available (Bird and Caskey, '43; Ellis et al., '45), it was a poor source of phosphorus in these studies. Mortality was excessive in both lots receiving this compound, especially in the lot which received 0.4% phosphorus. Growth at the lower level was very poor and all surviving chicks developed severe rickets. In the lot receiving 0.8% phosphorus, survival, growth, and bone ash values were better, but still markedly inferior, and all chicks exhibited gross rickets. These results indicate that some of the phosphorus in vitreous calcium metaphosphate is utilized by the chick but that its comparative availability is very poor.

The alkali metaphosphates, vitreous  $\text{NaPO}_3$ , and crystalline  $\text{KPO}_3$ , are soluble compounds which might be expected to be available biologically if the animal organism is able to metabolize metaphosphate. The sodium metaphosphate is com-

pletely water soluble while the potassium metaphosphate is 6.2% water soluble and 93.5% soluble in aqueous HCl. In these studies, however, the  $\text{NaPO}_3$  was very poorly utilized. The few chicks receiving this compound which survived the 4-week experimental period grew very little and were in a very weakened rachitic condition. Shelling ('32) concluded that sodium metaphosphate is biologically inert in the rat; however, our results show that it is very slightly used by the chick but is considerably less available than vitreous calcium metaphosphate. The other alkali metaphosphate studied,  $\text{KPO}_3$ , was virtually unavailable. No chicks survived the experimental period; however, survival on the higher level was slightly longer than on the unsupplemented basal diet. *Beta* and *gamma* calcium metaphosphates were completely unavailable.

A crude calcium phytate, purchased from a commercial source, was tested for availability in these studies. It is not necessarily exactly comparable to the product as it occurs in natural feedstuffs. The calcium phytate was almost completely unavailable under the conditions of this experiment in which 50 units of pure vitamin  $\text{D}_3$  were supplied per 100 gm of diet. No chicks survived the experimental period, but survival was a little better than on the unsupplemented basal diet. These results confirm those of Lowe, Steenbock and Krieger ('39) who found that the addition of phytin to a low phosphorus basal diet resulted in no increase in calcification, and those of Heuser and coworkers ('43) who concluded that a reasonable amount of non-phytin phosphorus must be included in chick rations.

Reynolds, Hill and Jacob ('44) proposed solubility in 0.4% HCl as a test for availability of different phosphorus supplements in the absence of information from actual feeding experiments. It is interesting to compare the solubility data for the supplements used in these experiments with the biological availability of the same materials. On the basis of the results reported in this paper, it would seem that solubility in 0.4% HCl has a limited usefulness in estimating availability

for animals. It can readily be seen that not a single one of the compounds which are relatively insoluble in this reagent is available for the chick. On the other hand, a comparative solubility cannot be taken as an indication of availability. Thus, the rather soluble *alpha*, *beta*, and *gamma* calcium pyrophosphates are completely unavailable biologically. The alkali metaphosphates constitute another example of soluble compounds which are not available. It appears, therefore, that solubility tests have only a negative value in that they may be used to eliminate certain insoluble compounds, but cannot be used to predict availability. It is difficult to understand why compounds which are comparatively soluble under conditions found in the digestive tract of animals should exhibit so little availability. It is to be remembered, however, that crystalline phosphates are capable of existing in a variety of polymerized forms (Hendricks, '44). When metaphosphates go into solution, covalent bonds as well as electrovalent forces must be broken. This results in particles of various sizes breaking off in the dissolving medium. It seems probable, therefore, that the type of sol may have much to do with determining the availability of the different phosphates. If the material presented to the absorbing membrane is in the form of hydrated micelles rather than ions in solution absorption would be impossible.

#### SUMMARY

The comparative availability of a number of common phosphorus supplements and pure phosphate compounds has been studied by adding them singly to a basal chick diet very low in phosphorus. When used to raise the phosphorus content of the basal diet to 0.4%, the orthophosphates, including  $\text{KH}_2\text{PO}_4$ , pure *beta* tricalcium phosphate and reagent grades of mono-, di-, and tricalcium phosphate, were excellent sources of phosphorus and slightly more available than steamed bone meal. At the same phosphorus level, the defluorinated superphosphate and defluorinated phosphate rock products used in



these experiments were good sources of phosphorus, but less available than the pure orthophosphates or steamed bone meal.

When used to raise the phosphorus content of the diet to 0.8%, the defluorinated superphosphate and defluorinated phosphate rock, both fused and calcined, were of equal value and were nearly as effective as pure *beta* tricalcium phosphate or steamed bone meal.

Crystalline calcium acid pyrophosphate was well utilized by the chick, while vitreous calcium metaphosphate was poorly utilized and vitreous sodium metaphosphate was only slightly available. Potassium metaphosphate, *beta* and *gamma* calcium metaphosphate, *alpha*, *beta*, and *gamma* calcium pyrophosphate, and crude calcium phytate were virtually unavailable. Comparison of the biological availability of the products used in these experiments with their solubility in 0.4% HCl shows that the solubility test is useful only in that insoluble compounds may be eliminated. The results also show that there was no difference in the availability of the different crystalline forms of a given chemical compound.

Severe phosphorus deficiency in the young chick results in early loss of appetite, weakness, and death within a period of 10 to 12 days.

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# AVAILABILITY OF RIBOFLAVIN OF ICE CREAM, PEAS, AND ALMONDS JUDGED BY URINARY EXCRETION OF THE VITAMIN BY WOMEN SUBJECTS<sup>1</sup>

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Information that will aid in distinguishing between the total content of specific vitamins in food materials and the relative amounts of those vitamins which can be utilized by the human subject should lead to a better understanding of how to attain adequate nutrition. Studies designed to determine the relative availability of vitamins from food sources have yielded a variety of findings. Fresh yeast (Parsons and associates, '45) contains an appreciable amount of thiamine which cannot be utilized by man. The extent to which biotin may be absorbed from liver and yeast apparently differs (Gardner et al., '46). Ascorbic acid seems readily available in several foods rich in this vitamin, as indicated by studies in which red raspberries, tomatoes, cabbage, apples, or papayas and guavas served as the test foods (Todhunter and Fatzer, '40; Clayton and Borden, '43; Hauck, '45; Anders,

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'46; Hartzler, '45). These observations make it apparent that knowledge of the vitamin content of the diet is only 1 step toward knowing whether the individual consuming it is well-fed.

Melnick and coworkers ('45) have suggested a human bioassay technic for the determination of the physiological availability of several water-soluble vitamins. The method depends upon the establishment of a constant urinary excretion of the particular water-soluble vitamin under study by normal subjects ingesting an adequate diet providing an excess of the vitamin in question. Then, when extra amounts of the pure vitamin are added to the diet, increased quantities are promptly excreted in the urine. The availability of a vitamin present in various food sources can then be measured by comparing the rise in the urinary output of the vitamin when the nutrient is supplied as a pure chemical and as a test food.

The technic proposed by Melnick and associates has been used in this study and results are reported on the relative availability of riboflavin present in 3 foods.

## EXPERIMENTAL

### *Subjects*

Eight women considered to be in good health participated in the experiment. Data concerning their age, height, and weight are given in table 1. The subjects continued their usual activities, proceeding with their graduate studies, assisting in the research laboratory, or teaching.

TABLE 1  
*Age, height and weight of subjects.*

SUBJECT	AGE	HEIGHT	WEIGHT	SUBJECT	AGE	HEIGHT	WEIGHT
	<i>yr.</i>	<i>inches</i>	<i>lb.</i>		<i>yr.</i>	<i>inches</i>	<i>lb.</i>
HW	21	67	125	EW	26	63	110
HB	32	63	123	GB	19	62	115
RM	29	66	134	MC	20	60	125
SK	23	64	126	GE	37	67	135

*Experimental plan*

Each subject served as her own control during a series of 5 experiments. In period I the magnitude and constancy of the urinary excretion of riboflavin were determined when the subjects ate the same mixed diet for a period of 12 days. In 4 subsequent 3-day periods the relative availability of the crystalline vitamin and that of the riboflavin in vanilla ice cream, frozen green peas and almonds were measured (periods II, III, IV, V).

During each study the subjects quantitatively consumed a weighed diet which provided approximately 2.4 mg of riboflavin daily. In each 3-day test period, the first day served as an adjustment interval and no urine was collected. On the second day, a 24-hour specimen of urine was collected to establish the amount of riboflavin excreted when the basal diet was consumed. On the third day, 1 mg of riboflavin was added to the basal diet either in the form of a solution of pure riboflavin or as that quantity of test food that contained 1 mg of the vitamin. The supplement was divided into 2 equal portions; one was taken at 10 A.M., and the other at 2:30 P.M. A second 24-hour specimen of urine was collected on the third day to determine the quantity of riboflavin excreted as a result of the administration of the supplement. The experimental days started at 7 A.M.

Each metabolism study was preceded by a 7-day preliminary period. During this time the subjects were allowed to select their own diets except that each was asked to consume sufficient protein from specified protein-rich foods to provide at least 75 gm daily. Since earlier studies had indicated that certain subjects were consuming too little protein, this precaution was found necessary to avoid a temporary storage of both nitrogen and riboflavin when the weighed diet was ingested later. The practice of consuming generous amounts of protein during the preliminary period prevented any further evidence of unusual retention of riboflavin.

During the first 6 days of the preliminary period, all subjects ingested a capsule containing 2 mg of riboflavin. No

food was consumed after 8 P.M. of the evening preceding the experimental studies. No medications were allowed during any portion of the investigation. The water intake was kept uniform during all collection periods.

One subject (GE) repeated the procedure outlined for period II for a total of 4 trials to determine the amount of variation which occurred in the urinary excretion of riboflavin when 1 mg of the pure vitamin supplemented the basal diet.

### *Composition and preparation of the basal diet*

The basal diet (table 2) was planned not only to meet the recommended allowances of the Food and Nutrition Board of the National Research Council ('45) but to provide a surplus of riboflavin. Canned products were purchased in sufficient amounts to cover the needs of the entire study.

TABLE 2  
*Composition of basal diet.*

Breakfast			
Orange juice <sup>1</sup>	150 gm	Milk	400 gm
Whole wheat bread	20 gm	Sugar	10 gm
Cornflakes	15 gm	Butter	10 gm
Dinner			
Lima beans <sup>1</sup>	100 gm	Milk	200 gm
Round steak	100 gm	Peaches <sup>1</sup>	150 gm
Whole wheat bread	20 gm	Peanut butter cookies	40 gm
Supper			
Rice (dry wt.)	30 gm	Apple sauce <sup>1</sup>	150 gm
Round steak	100 gm	Whole wheat bread	20 gm
Tomatoes <sup>1</sup>	150 gm	Butter	20 gm
Peanut butter cookies	40 gm	Sugar	15 gm
		Milk	200 gm
Lunch			
(Before 9 P.M.)			
		Peanut butter cookies	40 gm
<i>Estimated composition:</i>			
Protein	97 gm	Thiamine	1.4 mg
Fat	138 gm	Niacin	24.1 mg
Carbohydrate	282 gm	Ascorbic acid	110.0 mg
Calories	2758	Riboflavin	2.1 mg

<sup>1</sup> Used in the canned form.

Specially ground lean round of beef, which was purchased in 50-lb. quantities was mixed, wrapped as 100 gm patties, frozen and held at  $-5^{\circ}\text{F.}$  until needed. Whole wheat bread from 1 baking was purchased in an amount to exceed the needs of 1 experimental study and it was held at  $-5^{\circ}\text{F.}$

A lot of milk adequate for the entire series of studies was frozen in the college market milk laboratories after the usual pasteurization and homogenization. It was likewise held at  $-5^{\circ}\text{F.}$  until needed. Butter was supplied from 1 churning. Peanut butter cookies were prepared in the laboratory, standardized procedures of mixing and baking being followed.

In preparing the food to be used for each metabolism study, quantities of canned products sufficient for the experiment were opened, thoroughly mixed, and stored in sterilized jars in a refrigerator. All foods were weighed directly into the dishes in which they were served. Cooked foods were carefully heated so that both the length and degree of heating were kept constant. Precautions were taken at all times to avoid loss of riboflavin due to exposure to sunlight.

The 8 subjects consumed the same total amount of food daily and no refusals were encountered.

### *Supplements*

It was recognized that the response of the subjects might be altered by the fact that the ice cream, frozen green peas, and almonds selected as the first test foods contributed different quantities of carbohydrate, fat, and protein when consumed in amounts to provide 1 mg of riboflavin. Investigation of the influence of adding riboflavin-free sources of carbohydrate or fat to the basal diet indicated that extra amounts of either nutrient produced a change in the urinary excretion of riboflavin (Walker, '47). This was particularly true when fat served as the supplement. Increasing the protein by supplementing the diet with vitamin-free casein did not alter the urinary excretion of riboflavin.

To maintain the same daily total intake of carbohydrate, protein, and fat throughout all phases of each study the basal



diet was modified to include vitamin-free sources of these nutrients. Eighty gm dextrose, 100 gm butter<sup>2</sup> and 50 gm vitamin-free casein were needed to provide an excess of these nutrients above the quantities that would be encountered in supplying 1 mg of riboflavin from any of the 3 test foods. The sucrose, butter, and peanut butter cookies of the original basal diet were omitted to prevent an excessive intake of calories.

Each test food was assayed for riboflavin, total lipids, and nitrogen. Carbohydrate was estimated from published values (U. S. Bur. Human Nutrition, '45). The composition of the test foods is given in table 3.

TABLE 3  
*Composition of test foods.*

FOOD	WEIGHT CONSUMED	RIBOFLAVIN	CARBO- HYDRATE	PROTEIN	FAT
	<i>gm</i>	<i>mg</i>	<i>%</i>	<i>%</i>	<i>%</i>
Ice cream	377	1	20.9 <sup>1</sup>	4.1	11.2
Green peas	714	1	12.0 <sup>1</sup>	5.1	0.2
Almonds	112	1	6.5 <sup>1</sup>	22.8	54.0

<sup>1</sup> Available carbohydrate.

The supplement of pure riboflavin was consumed as a solution of the vitamin. The ice cream was prepared in the ice cream laboratory of the dairy industry department and all details of its composition were known. Frozen green peas were thawed slightly, then thoroughly mixed, and refrozen. They were heated for 30 minutes in an oven regulated at 425°F. before serving and were considered "done" but not overcooked. Almonds were served whole as the roasted unblanched nut meat.

#### *Collection of urine*

The 24-hour samples of urine were collected in amber bottles containing 50 ml of glacial acetic acid. During the

<sup>2</sup> The butter used contained 30  $\mu$ g riboflavin per 100 gm.

day all specimens were refrigerated at 36°F.; at night they were held at room temperature. The urine was diluted to a volume of 1500 ml. An aliquot of the diluted 24-hour samples was refrigerated until all analyses could be completed, which was within 10 days of collection in all cases.

### *Analytical methods*

An aliquot of the diet was prepared on the first and last days of the 12-day period (period I) and on the second day of each 3-day study. The riboflavin content of the diet as well as that of all test foods was determined by the microbiological method (Snell and Strong, '39; Strong and Carpenter, '42). The riboflavin content of the urine was determined by a modification of the fluorometric method described by Najjar ('41). Corrections for non-riboflavin fluorescent compounds were made by exposing the samples to bright sunlight for at least 10 hours to destroy the riboflavin. Nitrogen was determined by the Kjeldahl-Gunning procedure and the factor 6.25 was used for the conversion of nitrogen to protein. Total lipids were determined by continuous extraction with ether for 72 hours.

## EVALUATION OF METHOD AND RESULTS

### *Daily urinary excretion of riboflavin*

The quantity of riboflavin excreted daily when the subjects consumed the basal diet supplying approximately 2.4 mg of riboflavin per day was surprisingly constant for the 12 days of period I. The day-by-day variation encountered was 11% or less in all subjects except HB and GE. The data are given in table 4. In 6 of the 8 subjects the amount of riboflavin excreted ranged from 770 to 1090  $\mu$ g per day, equivalent to 33 to 43% of the intake. Subject HW showed a characteristically high excretion of the vitamin, 55% of the ingested riboflavin being eliminated in the urine. In contrast, subject GE excreted approximately 25% of the riboflavin of the diet.

The total daily excretion of riboflavin by the majority of the subjects agreed closely with that reported by Brewer et al. ('46) for college women ingesting approximately 2.7 mg of riboflavin daily. The excretion of the vitamin also approached the upper range of values reported by Keys and associates ('44) and by Melnick and coworkers ('45) for men

TABLE 4

*Daily urinary excretion of riboflavin when the basal diet was ingested.*

SUBJECT	HW	HB	RM	SK	EW	GB	MC	GE
	mg	mg	mg	mg	mg	mg	mg	mg
Riboflavin excreted								
Day 1	1.25	1.06	0.83	0.77	1.07	0.92	1.09	0.68
2	1.25	1.01	0.82	0.82	1.04	0.86	0.99	0.66
3	1.26	1.02	0.82	0.78	1.03	0.82	0.95	0.63
4	1.34	0.99	0.81	0.77	1.03	0.85	0.97	0.66
5	1.38	0.89	0.82	0.79	1.01	0.82	0.98	0.56
6	1.36	0.89	0.82	0.80	1.01	0.81	0.96	0.56
7	1.38	0.92	0.81	0.80	1.01	0.82	0.96	0.55
8	1.35	0.94	0.80	0.78	0.99	0.78	0.90	0.58
9	1.36	0.94	0.81	0.79	1.01	0.82	0.92	0.58
10	1.30	0.97	0.77	0.79	0.97	0.82	0.91	0.55
11	1.28 <sup>1</sup>	0.97 <sup>1</sup>	0.77 <sup>1</sup>	0.79 <sup>1</sup>	1.03 <sup>1</sup>	0.86 <sup>1</sup>	0.90 <sup>1</sup>	0.57
12	1.25	1.01	0.77	0.83	0.98	0.81	0.89	0.58
Per cent variation <sup>2</sup>	10.2	13.4	6.5	7.8	7.2	10.2	11.0	20.0
Per cent of intake excreted (average)	55	40	34	33	43	35	40	25

<sup>1</sup> 2 mg riboflavin administered in a retention enema the morning of the eleventh day.

<sup>2</sup> First day excluded in estimation of per cent variation.

who consumed 2.5 to 2.7 mg riboflavin per day. The amount of vitamin excreted by subject GE agreed with the lowest values reported by Melnick and others.

The data in general indicated that all 8 women were suitable subjects for participation in this particular type of metabolic study.

It may be of interest to workers in other laboratories to know that the use of a uniform water intake did not assure a uniform output of urine. Since these studies extended over several months, wide differences were nevertheless encountered in weather conditions which greatly altered perspiration losses. It was noted, also, that the urine volumes were smaller just preceding menstruation although no changes were observed in the quantity of riboflavin excreted at these times. From the studies of Johnson ('46) it would seem that the excretion of riboflavin was not influenced by the volume of urine unless extremely small or excessively large amounts of urine were eliminated.

*Influence of administration of riboflavin by retention enema upon the urinary excretion of the vitamin*

Considerable interest has been aroused by the possibility that the amount of riboflavin excreted in the urine may be influenced by the synthesis of riboflavin in the intestinal tract. Data reported by Najjar and associates ('44) support this idea. A more recent investigation by Alexander and Landwehr ('45) on the role of fecal thiamine in human nutrition indicates that this vitamin is not absorbed from the lower portion of the intestine and that the urinary excretion of the vitamin does not change if physiological amounts of thiamine are administered by retention enema.

Since it was possible that the ingestion of the various test foods might alter the intestinal flora and in turn influence the urinary excretion of riboflavin due to absorption of the vitamins synthesized by the flora, the effect of administering physiological amounts of riboflavin by retention enema upon the urinary output of the vitamin was studied. Two mg of riboflavin were given by retention enema on the morning of the eleventh day of period I. The vitamin was retained by the various subjects from 10.5 to 39 hours and in no case was there a measurable influence on the excretion of riboflavin in the urine (table 4).

The importance of bacterial synthesis in providing available riboflavin to human subjects is therefore questioned. Although the introduction of test foods into the diet may have altered the total riboflavin synthesized in the intestinal tract, it would appear from the findings of this study that this vitamin was not absorbed.

*Influence of adding 1 mg of pure riboflavin*

In the availability studies reported by others (Melnick et al., '45; Oser, Melnick and Hochberg, '45), in which vitamin concentrates or the pure chemical were tested, larger supplements were used than is practical when foods supply the vitamin. It was necessary, therefore, to determine whether a smaller supplement would create sufficient rise in riboflavin excretion in the urine to constitute a reliable test of differences in availability. A dose of 1 mg was chosen for testing the response of the subjects, since quantities of foods required to supply this amount of the vitamin could be consumed without difficulty. To avoid the need for eating large amounts of food at one time as well as to make use of the possibility that a divided dose of riboflavin might permit more efficient absorption of the vitamin, the supplement was divided into 2 equal portions.

Data given in tables 5 and 6 show that the increment in the urinary excretion of the vitamin induced by the supplementation of the diet with riboflavin was surprisingly high. The per cent of the test dose returned varied widely (31 to 67%) in the different subjects. The average return for the 8 subjects was slightly over 42%. This percentage return was not much different from that observed by Melnick and associates for men ingesting 10 mg of riboflavin immediately after the noon meal.

Thus it appears that adequate stimulation of riboflavin excretion was produced by the addition of two 500  $\mu$ g supplements, and that the technic was practical for testing the relative availability of riboflavin from food sources. The

effect upon the efficiency of absorption of ingesting frequent small amounts of riboflavin rather than 1 large supplement is being studied at the present time.

To check further the acceptability of the 1 mg quantity of riboflavin as the test dose, subject GE repeated the procedure outlined for period II during 4 consecutive months. Each test included the standardized preliminary period and the same basal diet. It may be noted from table 5 that this subject responded very uniformly, the per cent of the test dose returned ranging only from 32 to 38.

TABLE 5

*Reproducibility of urinary excretion of riboflavin following supplementation of 1 mg pure riboflavin (subject GE).*

PERIOD	RIBOFLAVIN SUPPLEMENT	BASAL EXCRETION	AFTER TEST DOSE	TEST DOSE RETURNED
	mg	μg	μg	%
I (December)	1	484	839	36
II (January)	1	593	917	32
III (February)	1	538	913	38
IV (March)	1	646	1017	37

*Availability of riboflavin of ice cream,  
green peas, and almonds*

When ice cream served as the test food a marked rise occurred in the quantity of vitamin appearing in the urine (table 6). The increment corresponded closely to that produced by the addition of 1 mg of the pure vitamin. Six of the 8 subjects responded similarly to the addition of riboflavin as the pure vitamin or as the test food, while 2 subjects (SK and GB) showed a slightly smaller excretion of the vitamin when riboflavin was derived from the food. The average percentage of test dose returned by the 8 subjects (38.5)

indicated that the riboflavin of ice cream was present in a physiologically available form.

When either frozen green peas or almonds furnished the 1 mg supplement, all 8 subjects excreted less riboflavin than they had on 2 previous occasions when either the pure vitamin or ice cream served as the source of the nutrient. These differences in excretion of the vitamin indicated that a smaller percentage of the total riboflavin occurring in peas or almonds was available.

TABLE 6  
*Availability of riboflavin of 3 foods.*

SUBJECT	PERIOD II 1 MG RIBOFLAVIN (PURE VITAMIN)	PERIOD III 1 MG RIBOFLAVIN (ICE CREAM)	PERIOD IV 1 MG RIBOFLAVIN (GREEN PEAS)	PERIOD V 1 MG RIBOFLAVIN (ALMONDS)
Per cent test dose excreted				
HW	39	33	20	5
HB	46	47	20	6
RM	43	43	18	15
SK	67	56	32	40
EW	38	34	20	22
GB	45	35	11	21
MC	31	28	9	3
GE	32	32	12	20
Average	42.6	38.5	17.7	16.5
Availability of riboflavin in ice cream =	$\frac{38.5}{42.6}$	$\times 100 = 90.4 \pm 11\%$		
Availability of riboflavin in green peas =	$\frac{17.7}{42.6}$	$\times 100 = 41.5 \pm 11\%$		
Availability of riboflavin in almonds =	$\frac{16.5}{42.6}$	$\times 100 = 38.7 \pm 11\%$		

It should be brought out, however, that had the peas or almonds been used in another form, i.e., as the puréed vegetable or as almond paste, a higher percentage of the riboflavin present might have been absorbed.

While considerable individual difference was observed between subjects as to the percentage of test dose returned when 1 mg of pure vitamin was ingested, the subjects were fairly consistent in their response to the addition of the

vitamin in the form of food. It is interesting to note that SK, who excreted 67% of the test supplement when pure riboflavin was consumed always excreted the highest percentage of the test dose of any of the subjects regardless of the source of the vitamin, and that MC consistently excreted the smallest fraction of the added riboflavin.

No explanation can be given at this time for the striking differences in the apparent availability of riboflavin in the 3 food sources. It is thought that these differences may be due to variations in absorbability. Possibly the presence of bound forms of the vitamin in peas or in almonds may account for part of the difference. It may be found that the presence of certain other constituents in the intestinal tract at the time that riboflavin absorption takes place limits the quantity of the vitamin which is available. Apparently these differences cannot be explained by variations in acid-base balance since Johnson ('46) observed no change in urinary output of riboflavin following wide fluctuations in urinary pH.

Additional studies are planned to determine some of the factors which may influence the availability of riboflavin of foods.

#### SUMMARY

Eight healthy women subjects participated in 5 experimental studies in which the relative availability of the riboflavin present in ice cream, frozen green peas, and almonds was determined. During a 12-day period when a weighed diet containing 2.4 mg of riboflavin was ingested, the urinary excretion of riboflavin was strikingly constant.

Administration of 2 mg of riboflavin by retention enema on the morning of the eleventh day produced no change in the amount of riboflavin which appeared in the urine.

A marked rise in the concentration of riboflavin of the urine occurred for all subjects when two 500  $\mu$ g portions of pure riboflavin supplemented the basal diet. The percentage of the test dose returned varied from 31 to 67 with an average value of 42% for the 8 subjects.



When a supplement of 1 mg of riboflavin was supplied in the form of ice cream, the increment in urinary riboflavin was comparable to that produced by the pure vitamin, indicating that the riboflavin of ice cream was nearly as available as that of the pure vitamin.

When either frozen green peas or almonds furnished 1 mg of the supplementary vitamin, considerably less riboflavin was excreted in the urine than when the pure vitamin was administered. Less than one-half as much riboflavin appeared to be absorbed from these foods as had been absorbed from ice cream or the pure vitamin.

Investigations are under way to determine some of the factors which influence the availability of riboflavin in foods.

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# COMPARISON OF VITAMIN A LIVER STORAGE FOLLOWING ADMINISTRATION OF VITAMIN A IN OILY AND AQUEOUS MEDIA

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It has been previously observed in this laboratory that vitamin A when administered in aqueous media is absorbed much more readily than when administered in oil. This difference is especially important in conditions where impaired intestinal absorption exists, as in the celiac syndrome, and in the normal newborn infant (Kramer, Sobel and Gottfried, '47; unpublished results of Sobel, Besman and Kramer).<sup>1</sup> The criterion in absorption was the change in vitamin A blood serum levels following the administration of the vitamin A-containing preparation (Kramer, Sobel and Gottfried, '47; Sobel, Besman and Kramer<sup>1</sup>; May, Blackfan, McCreary and Allen, '40).

The question arose as to whether or not an increase in the blood level actually represents increased absorption. Since metabolic studies are open to the objection that vitamin A may be destroyed in its passage through the intestine itself (Sherman, '47), we undertook to determine vitamin A absorption by the liver storage test.

<sup>1</sup> Presented before the Baskerville Chemical Society of the College of the City of New York, April 24, 1947; before the New York Pediatric Research Club, New York City, April 2, 1947; and in part before the American Society of Biological Chemists, Chicago, May 22, 1947.

## EXPERIMENTAL

The liver storage test was employed using essentially the technique of Lemley, Brown, Bird and Emmett ('47). The rats used were from an inbred Wistar strain kept on the stock diet of Bills et al. ('31). Fifteen-day-old rats, together with the mother, were put on the U.S.P. XII Vitamin A Test Diet<sup>2</sup> with the following percentage composition: vitamin-free casein 18, U.S.P. no. 2 salt mixture 4, irradiated yeast 8, starch 65, and hydrogenated vegetable oil mixture consisting primarily of hydrogenated cottonseed oil<sup>3</sup> 5. At 20 days of age, the weanling rats were removed to experimental cages and the U.S.P. diet and distilled water given ad libitum. On this regime deficiency followed in 25-30 days, as determined by failure to gain weight for 3 successive days or by the presence of xerophthalmia.

Following the vitamin A depletion period, each litter was split into groups, each group receiving one of the preparations which were being used for comparison. The vitamin A-containing preparations were administered on 3 successive days, 0.2 ml being given each time through a stomach tube, employing the technique of Shay and Gruenstein ('46). The stomach tube is essential since the rats will not take aqueous dispersions by simple oral feeding. On all preparations administered, the vitamin A content was determined at the beginning and at the end of each experiment, employing the method of Oser et al. ('43).

At the beginning of the fifth day, the rats were sacrificed by cutting the carotid arteries and jugular veins. The vitamin A content of the blood serum was determined by the GDH (activated glycerol dichlorohydrin) method of Sobel and Snow ('47). Each whole liver was analyzed separately, immediately after removal.

The method of liver analysis was simplified in order to shorten the procedure and to increase the reliability of results. The conventional method of analysis, as exemplified by Oser

<sup>2</sup> Obtained from General Biochemicals, Inc.

<sup>3</sup> Primex.

et al. ('43), requires a total of 10 extractions and washings in separatory funnels, and results in a large volume of extract. It also requires the preparation of freshly redistilled ethyl ether. The method of Gallup and Hoeffer ('46) where a 1-gm sample is punched out of the liver as an aliquot, is open to 2 objections: (1) the assumption of a representative sample is invalid (Sanford and Bucher, '46), and (2) it is difficult to remove and weigh a 1-gm sample without loss of weight by evaporation. In addition the 1 extraction with petroleum ether recommended by Gallup and Hoeffer ('46) may sometimes be incomplete.

In our analytical procedure each whole liver was finely minced and placed in a flask with 0.75 ml 60% KOH and 7.5 ml 95% aldehyde-free ethyl alcohol per gram of liver. The liver saponification mixture was refluxed on a hot water bath for 1 hour, or until the liver was completely dissolved, using ground-glass connections throughout. The resulting solutions were made up to 50 or 100 ml volume with 95% aldehyde-free ethyl alcohol in volumetric flasks, depending upon the original size of the liver.

From these solutions 1 ml aliquots were removed and placed in a glass-stoppered test tube, 1 ml of distilled water was added and the mixture extracted while being agitated by a shaking machine with 2 ml redistilled petroleum ether (B.P. 30°-60°C.) for 10 minutes. The tubes were then centrifuged at low speed for 30 seconds and the supernatant liquid removed by aspirating with a fine-tipped dropper. The extraction was repeated 2 more times using 1 ml of petroleum ether each time. The vitamin A content of the combined extracts was determined as described by Kramer et al. ('47) for the petroleum ether extract of blood serum. By this method as many as 10 simultaneous determinations may be completed by 1 person in 2 hours following saponification. The proposed method represents a step towards greater precision and reliability in single rat liver analysis, since duplicates and recoveries may be obtained, as contrasted to the methods where the whole liver is used for a single analysis. As shown in table 1 good agreement is demonstrated between this method

and that of Oser et al. ('43) which was carried out on 20 to 40 ml of digest representing 40% of the liver.

TABLE 1

*Comparison of liver vitamin A analysis by the proposed method and by the method of Oser et al. ('43).*

LIVER NO.	PROPOSED METHOD <sup>1</sup>	METHOD OF OSER ET AL. <sup>1</sup>	LIVER NO.	PROPOSED METHOD <sup>1</sup>	METHOD OF OSER ET AL. <sup>1</sup>
1	130	123	8	334	336
2	148	138	9	284	295
3	209	217	10	152	153
4	395	410	11	210	232
5	356	366	12	249	264
6	405	388	13	159	173
7	379	398			
			Mean value	262	269

<sup>1</sup> Expressed as total USP units of vitamin A per whole liver.

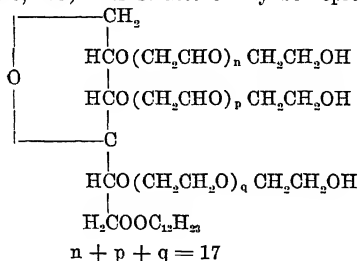
#### VITAMIN A ABSORPTION AS MEASURED BY LIVER STORAGE

##### *Unaponifiable fraction of fish liver oil in oily and aqueous media*

In these experiments, a commercial concentrate <sup>4</sup> was used. This is the unaponifiable fraction of fish liver oil containing about 800,000 to 1,000,000 U.S.P. units of vitamin A per gram. This material was diluted to the same degree (a) in maize oil, (b) in water containing 16% of the dispersing agent <sup>5</sup> sup-

<sup>4</sup> The Borden Co., New York.

<sup>5</sup> The dispersing agent used was sorbitan monolaurate polyoxyethylene derivative (Griffin, '45; Cordero, '45). Its structure may be represented by



This compound is an edible dispersing agent. A full description of its physical, chemical and physiological properties may be obtained from the Atlas Powder Company under the name of Tween 20.

plemented by other vitamins to prepare aqueous dispersion I,<sup>6</sup> and (c) in water containing 16% of the dispersing agent to prepare aqueous dispersion II.<sup>7</sup>

The results are shown in table 2 and summarized in table 3. There was about 3 times as much vitamin A in the livers of the animals receiving aqueous dispersions I and II as in those receiving the oily solution. The difference between the deposition effected by the 2 aqueous dispersions is negligible, that due to aqueous dispersion I being slightly higher. Thus, under these conditions, as measured by the vitamin A storage test, this vitamin is far better absorbed from aqueous media than from an oily solution. There was no obvious relationship between serum vitamin A levels and liver storage, except that animals deficient in this vitamin (negative controls) showed no measurable vitamin A in the serum. This lack of correlation is in agreement with the findings of several other investigators (McCoord and Luce-Clausen, '34; Nylund and With, '41; Leong, '41). Glover, Goodwin and Morton ('47) "showed that the plasma vitamin A levels are proportional to the concentrations of vitamin A alcohol in the livers, but are not proportional to the total liver store of vitamin A which consists mainly of esters."

*Unaponifiable fraction of fish liver oil and natural  
esters in maize oil*

The notable difference between the findings of the preceding experiment and those of Lemley, Brown, Bird and Emmett ('47) is that our percentage of vitamin A deposited in the liver in the case of the oily solution is only about one-third

<sup>6</sup> Aqueous dispersion I is called "Vifort." Each 0.6 ml contained in addition to the vitamin A, 1200 USP units of vitamin D, 1.8 mg of thiamine hydrochloride, 0.6 mg riboflavin, 0.3 mg pyridoxine hydrochloride, 60 mg of ascorbic acid, 3 mg of nicotinamide, 1.2 mg of calcium pantothenate and 132 mg of extract of rice polishings, and also contained choline, inositol and biotin vitamins.

<sup>7</sup> Aqueous dispersions I and II were similar to that previously used in children (Kramer, Sobel and Gottfried, '47) except that both contained 16% of dispersing agent instead of 11% in dispersion I and 20% in dispersion II. In the vitamin A absorption studies on newborn children (Sobel, Besman and Kramer, unpublished) both preparations contained 16% of the dispersing agent.



TABLE 2

*Comparison of vitamin A liver storage in rats given vitamin A in oily and aqueous media.*

Vitamin A values are expressed in USP units

MEDIUM	GROUP	NO. CASES	MEAN BODY WT.	AMT. VIT. A ADMIN.	AMT. ADM. PER GM WT.	MEAN LIVER DEPOSIT	% OF TOTAL DEPOSIT	SERUM VIT. A /100 ML
Vitamin A alcohol (Borden's concentrate) in maize oil and in aqueous dispersing media I and II								
Maize oil	A <sup>1</sup>	8	76.8	1250	16.3	123	9.8	75
	B	4	106.1	1090	10.3	129	11.8	98
Aqueous dispersion I	A	10	70.6	1250	17.7	355	28.4	88
	B	5	98.6	1155	11.7	362	31.4	99
Aqueous dispersion II	A	8	93.9	1390	14.8	385	27.7	123
	B	5	109.7	1275	11.6	366	28.8	87
Negative controls	A	3	109.3	....	...	10	...	0
Vitamin A alcohol (Borden's concentrate) and esters (Oleum Percomorph and distilled) in maize oil								
Vitamin A alcohol in maize oil	C <sup>1</sup>	2	88.0	1250	14.2	152	12.2	89
	D	3	57.8	1350	23.4	141	10.4	154
	E	3	61.7	2695	43.7	93	3.5	169
Oleum percomorph in maize oil	C	3	70.0	1250	17.9	124	9.9	66
Distilled ester in maize oil	D	3	51.0	1430	28.1	231	16.2	177
	E	3	62.0	2860	46.1	331	11.6	128
Vitamin A ester (distilled) in maize oil and in aqueous dispersing medium III								
Maize oil	F <sup>1</sup>	4	95.4	1215	12.7	217	17.9	246
	G	4	126.9	1220	9.6	160	13.1	111
Dispersion III	F	4	83.9	1215	14.5	465	38.3	166
	G	4	119.5	1195	10.0	349	29.2	103
Negative control	G	1	100.5	..	...	49		0

<sup>1</sup> Each letter represents corresponding litter groups.

that of the percentages reported by Lemley. This presented the possibility that the unsaponifiable fraction of fish liver oil is more poorly absorbed than other forms of vitamin A, such as the natural ester used by Lemley, Brown, Bird and Emmett ('47).

It has been observed by several investigators (Emmett and Bird, '37; Gray, Hickman and Brown, '40) that there is a difference between the biological assay value of vitamin A in

TABLE 3  
*Comparative efficacy of various vitamin A preparations.*

Vitamin A values are expressed in USP units

PREPARATION	NO. CASES	MEAN LIVER DEPOSIT	% OF TOTAL DEPOSIT	SERUM LEVELS/100 ML		
				Low	Medium	High
Unsap. fish oil in oil solution	17	131	10.7	41	92	169
Distilled ester in oil solution	11	200	15.7	79	152	287
Oleum percomorph in oil	3	124	9.9	49	62	89
Aqueous dispersion I	15	357	29.4	67	96	118
Aqueous dispersion II	13	378	28.1	62	96	188
Aqueous dispersion III	8	407	33.7	96	118	217
Negative controls	4	20	...	0	0	0

the free alcohol form and that of the esterified form. As shown in table 2, under the conditions of this experiment, there is a negligible difference between the efficacy of unsaponifiable fraction of fish liver oil concentrate and Oleum Percomorph at the same level of administration. However, it appears that the distilled natural ester is about 1.5 times as efficient as the unsaponifiable fraction of fish liver oil at this level. Litter E, although an atypical example because of a prolonged de-

iciency period (35 days), also shows a definite difference between the deposition of the unsaponifiable fraction and that of the natural ester. However, the maximum deposition attained with the natural ester is still only one-half of that reported by Lemley, Brown, Bird and Emmett ('47).

### *Distilled natural esters in oily and aqueous media*

In these experiments a distilled natural ester<sup>8</sup> containing about 400,000 U.S.P. units of vitamin A per gram and 0.5% tocopherols, was diluted to the same degree in maize oil and in water containing 40% of the dispersing agent<sup>9</sup> to prepare aqueous dispersion III. This high percentage of the dispersing agent was used in order to obtain a clear dispersion. The results are shown in table 2. There was about 2.2 times as much deposition with the aqueous dispersion as with the oily solution. In the case of the aqueous dispersion of the natural ester, the absolute percentage of deposition was actually higher than the maximum deposition reported by Lemley, Brown, Bird and Emmett ('47) for oily solutions under optimum conditions.

### *The absorption of vitamin A as measured by liver storage of the various preparations*

The results of all experiments are summarized in table 3. It may be readily observed that the aqueous dispersions in general showed a far higher percentage of absorption as measured by liver deposition than the same vitamin A-containing preparations diluted in maize oil. Among the oils, the distilled esters gave higher per cent deposition than the unsaponifiable fraction of fish oil.

### DISCUSSION

The present experiments show that, in addition to those factors mentioned by Clausen ('43) as affecting the ability

<sup>8</sup> No. 11537, from Distillation Products, Inc.

<sup>9</sup> See footnote 5, page 228.

to absorb vitamin A, the medium of aqueous dispersion must also be considered an important factor. The probable explanation is that the size of the individual vitamin A-containing particles is in itself an important factor in absorption. The importance of particle size was indicated by Frazer et al. ('44). They have shown that olive oil in the intestine of the rat is dispersed to a fine particle size less than  $0.5\ \mu$ . Furthermore, in rats fed paraffin in an aqueous dispersion containing particles of an average size of less than  $0.5\ \mu$ , the paraffin is absorbed by the intestine as well as olive oil dispersed to an equal particle size in water, whereas *paraffin itself* is not absorbed at all by the intestine. Lundbaek and Maaløe ('47) however, were unable to repeat the paraffin experiments.

It is possible, in addition to the particle size property, that the dispersing agent itself plays a part in improved absorption by acting as a wetting agent, changing the inter-facial tension or changing the surface of the intestine or the channels through which the vitamin A is absorbed. In a paper published during the course of this investigation (Halpern, Biely and Hardy, '47) where vitamin A was dispersed in water by another agent (1% "methocel," 0.05% mixed tocopherols and 0.1% lecithin), it was shown by the bio-assay value as measured by the growth of chicks, that the aqueous dispersion was far more effective than the oily solution.

Polskin ('40) has shown that in the chick there is increased liver deposition when lecithin is added to the vitamin A supplement, while Slanetz and Scharf ('45) have shown the same effect for the rat. These effects may be due to the well-known emulsifying property of lecithin rather than to some specific effect of lecithin *per se*.

In view of these findings one can state with fair assurance that the sharp rise in the vitamin A tolerance curve for blood serum for aqueous dispersions, as opposed to the flat curves obtained with oily solutions (Kramer, Sobel and Gottfried, '47; unpublished results of Sobel, Besman and Kramer<sup>10</sup>)

<sup>10</sup> See footnote 1, page 225.

(in cases of impaired absorption such as the celiac syndrome and the normal newborn infant), actually represents improved absorption and retention of vitamin A rather than merely a temporary high blood level.

Until all of the vitamin A administered is accounted for, it is difficult to completely eliminate stability, sparing action or both as factors. Such an explanation, however, would hardly account for an average maximum rise in serum vitamin A of 69  $\mu\text{g} \%$  with oil and 636  $\mu\text{g} \%$  with the same amount of vitamin A in water<sup>11</sup> (Kramer, Sobel and Gottfried, '47). Furthermore, there is no evidence to indicate that aqueous dispersions of vitamin A are more stable than vitamin A in oils. Fish oils and distilled esters contain antioxidants and are considered more stable than the nonsaponifiable fraction of fish oil present in dispersion II. It is not likely that a sparing action of vitamin A could be demonstrated in the short period of these experiments. Lemley, Brown and Emmett ('47) have found no significant sparing action of vitamin E on liver storage under experimental conditions similar to ours. They were able to demonstrate a sparing action only when smaller daily doses were fed for 3 to 6 months.

The vitamin supplements in aqueous dispersion I<sup>12</sup> did not reduce the amount of liver storage as would be expected from the vitamin A tolerance tests in normal children reported by Kramer, Sobel and Gottfried ('47). However, it was later shown in unpublished experiments by Sobel, Besman and Kramer that when the per cent of dispersing agent<sup>13</sup> is identical in aqueous dispersions I and II<sup>12, 14</sup> (as was the case in these experiments) the improvement in vitamin A

<sup>11</sup> Vitamin A tolerances obtained with vitamin A as the alcohol in oil (Borden's concentrate in maize oil) and vitamin A as the ester in oil (Oleum Percomorph) were similar (unpublished results of Sobel, Besman and Kramer). Thus the differences between vitamin A tolerances obtained in aqueous and oily media are not due to the fact that the ester was given in oil and the alcohol in aqueous medium as suggested by Embree ('47).

<sup>12</sup> See footnote 6, page 229.

<sup>13</sup> See footnote 5, page 228.

<sup>14</sup> See footnote 7, page 229.

absorption when compared to oily solutions (as measured by the vitamin A tolerance test) is the same with both aqueous preparations.

The results of this experiment show a markedly lower percentage of liver deposition in the case of oily preparations than that reported by Lemley, Brown, Bird and Emmett ('47). This may be explained by one or more of several factors known to affect the degree of absorption in experiments of this type. Several investigators have reported that the type and condition of the oil used in the basic deficient diet have a marked effect on the bioassay of vitamin A (Lathbury and Greenwood, '34; Lease et al., '38; Harrelson and co-workers, '39). Foy and Morgareidge ('47) in testing the Guggenheim and Koch ('44) method of bio-assay found that when vitamin A was dissolved in sardine oil, the deposition was 50% of that which they obtained when the vitamin A was dissolved in maize oil. The oil used in their vitamin A deficient diet was maize oil instead of the hydrogenated cottonseed oil in our diet. It is conceivable that if they had used sardine oil in their vitamin A deficient diet, the sardine oil effect would have been even more marked. Another factor involved is the actual nature of the vitamin A deficient diet (Gridgeman, Lees and Wilkinson, '40).

The well-known effect of vitamin E on vitamin A absorption and storage has been repeatedly emphasized. The subject has been reviewed by Moore ('45).

It is also possible that the difference between our results and those reported by Lemley, Brown, Bird and Emmett ('47) may be due to the difference in the strain of rats used rather than to the dietary factors mentioned above.

Regardless of the underlying cause of the discrepancy, our rats had impaired absorption of vitamin A from oil as compared to the rats used by Lemley, Brown, Bird and Emmett ('47). Thus, the situation in some respects is analogous to that of children with celiac syndrome who have impaired absorption as compared to normal children (Kramer, Sobel and Gottfried, '47) and newborn infants who have impaired ab-

sorption as compared to older children (unpublished data of Sobel, Besman and Kramer). However, the important fact is that, on administration of aqueous dispersions of vitamin A, we obtained greater absorption, as measured by liver storage, than with oily solutions. This same difference in absorption from oily and aqueous media was also observed in children with impaired absorption as measured by the vitamin A tolerance test.

A salient point brought out by this observation is the variability of the results which may be obtained by using the liver storage bio-assay procedure proposed by Guggenheim and Koch ('44), depending upon the nature of the medium used for the administration of vitamin A.

#### SUMMARY

Vitamin A liver storage tests were used as the criterion for determining the relative absorption of vitamin A from oily and aqueous media. Young rats were placed on the U.S.P. Vitamin A Test Diet containing hydrogenated cottonseed oil. The deficient rats were fed the preparations for 3 successive days and sacrificed at the beginning of the fifth day for vitamin A analyses of liver and serum.

There was no definite relationship between serum levels and liver storage, except that vitamin A deficient animals gave low serum values.

Liver storage was 3 times as great in groups fed the unsaponifiable fraction of fish liver oil dispersed in water as compared with the same fraction administered in maize oil.

Liver storage of groups fed distilled natural esters in maize oil was about 1.5 times as great as that deposited in groups fed the unsaponifiable fraction in maize oil.

Distilled esters dispersed in water gave 2.2 times the deposit as compared to distilled esters in maize oil.

Apparently vitamin A is more effective when dispersed in aqueous media than in oily solutions. This indicates the importance of considering the nature of the diluent material in the biological evaluation of vitamin A.

## ACKNOWLEDGMENT

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# NIACIN EXCRETION IN THE RAT IN RELATION TO TRYPTOPHANE, PYRIDOXINE, AND PROTEIN CONTENT OF THE DIET<sup>1</sup>

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A considerable body of evidence has accumulated in recent years to indicate that the formation of niacin in the organism of the rat is dependent on the intake of tryptophane (Rosen, Huff and Perlzweig, '46; Singal et al., '46). The most probable explanation of this relationship is that niacin is actually synthesized from tryptophane. On the other hand, beginning with the work of Lepkovsky and coworkers ('43), it has become increasingly evident that pyridoxine is intimately concerned in the metabolism of tryptophane. We accordingly began a study of the influence of pyridoxine in the diet on the niacin excretion of rats. While this work was in progress, Rosen, Huff and Perlzweig ('47) and Schweigert and Pearson ('47) published the results of studies showing that excretion of niacin and N<sup>1</sup>-methylnicotinamide by rats, following administration of tryptophane, was greater in animals fed pyridoxine than in animals on diets deficient in this vitamin.

The results of our studies are in agreement with these conclusions, but show in addition that the effects of pyridoxine are more evident when the tryptophane is administered as the free amino acid. Singal and coworkers ('46) showed that an increase in the protein (casein) content of the diet did not increase niacin excretions; our results confirm this.

<sup>1</sup> The data are taken from a thesis presented by Grace H. Bell to the Graduate School of the University of Southern California in partial fulfillment of the requirements for the degree of Master of Science.

## METHODS

Rats of the University of Southern California strain were used. They were kept in large wire cages with screen bottoms or, for collection of urine, in individual metabolism cages. Urine was collected under toluene, or in bottles containing 3% acetic acid in amounts such that the final acetic acid concentration of urine and washings was about 2%. The preserved urines were stored in a refrigerator at 4°C. until analyzed. Niacin was determined on individual urines by microbiological assay with *Lactobacillus arabinosus* (U. S. Pharmacopoeia XII, first supplement revised), without acid hydrolysis. N<sup>1</sup>-methylnicotinamide was determined fluorometrically by the method of Huff and Perlzweig ('47), using aliquots of pooled specimens from 5 rats.

The basal diet used in all experiments had the following composition: protein 15%, sucrose 72%, celluloflour 4%, salts (Osborne and Mendel, '17) 4%, cottonseed oil<sup>2</sup> 3%, vitamin A and D oil mixture (6 parts cottonseed oil to 1 of superbiotol) 2%, choline chloride 150 mg, thiamine hydrochloride 2 mg, calcium pantothenate 4 mg, riboflavin 2 mg, and folic acid 0.5 mg per 100 gm diet. The proteins used were fish protein (Deuel et al., '45), vitamin-test casein,<sup>3</sup> and zein<sup>4</sup>. The fish protein was assayed for niacin and pyridoxine (Bird et al., '42), and no detectable amount of either was found. The zein, after acid hydrolysis, contained 0.6 mg of niacin and 0.3 mg of pyridoxine per 100 gm. To some diets, either protein or amino acids were added in excess of 15%; the sucrose percentage was decreased accordingly in these cases.

*Niacin excretion on diets without added tryptophane*

Ten male rats, weighing between 106 and 139 gm were fed the basal ration, deficient in pyridoxine, with 15% fish protein, for 2 weeks. They were then paired as to weight and food

<sup>2</sup> Wesson.

<sup>3</sup> General Biochemicals, Inc.

<sup>4</sup> Corn Products, Inc.

consumption. One member of each pair was continued on the basal ration; the other received the basal ration supplemented with 0.15 mg pyridoxine hydrochloride per 100 gm for the first week, 0.3 mg the second week, and 4.0 mg thereafter. The food intake of the animals fed pyridoxine was limited to that taken by the member of the pair on the ration lacking pyridoxine.

TABLE 1

*Food intake, weight changes, and niacin excretion of rats fed the basal diet with fish protein, with and without pyridoxine. Average values for 5 rats in each group.*

	GROUP <sup>1</sup>	TIME IN WEEKS					
		1	2	3	4	5	6
Pyridoxine intake,	+	0	0	14	26	405	362
μg/rat/day	—	0	0	0	0	0	0
Food intake,	+	12.3	10.2	9.6	8.7	10.0	9.0
gm/rat/day	—	12.3	9.9	9.3	9.0	10.0	9.0
Weight change,	+	+ 4.0	+ 2.4	— 0.5	+ 0.3	+ 3.2	+ 0.3
gm/rat/day	—	+ 2.9	+ 2.4	+ 0.2	+ 0.4	+ 2.0	+ 0.2
Niacin excretion,	+	42.6	46.0	56.3	42.6	43.8	53.6
μg/rat/day	—	29.6	37.5	43.5	32.2	37.5	37.5
N <sup>1</sup> -methylnicotin-							
amide excretion,	+	137.8	138.8	146.5	86.0	87.0	86.8
μg/rat/day	—	115.5	134.2	153.2	92.0	65.4	119.7
Total niacin <sup>2</sup> as	+	100	102.6	113.0	72.0	73.4	79
% of first week	—	100	118.2	135.7	86.0	72.2	107.9

<sup>1</sup> Group + received pyridoxine supplement during the third to sixth week of the experiment. The food intake of this group was restricted to that taken by group —, which received no pyridoxine.

<sup>2</sup> Niacin + N<sup>1</sup>-methylnicotinamide.

The results of this experiment are presented in table 1. It is evident that the addition of pyridoxine to the diet does not result in an increased output of niacin or N<sup>1</sup>-methylnicotinamide by rats maintained for 2 weeks on a diet lacking pyridoxine. The niacin excretion of the group receiving pyridoxine (+) was somewhat higher throughout than that of the deficient group (—), but there was no change in this

level when pyridoxine was added to the diet. No signs of pyridoxine deficiency, other than a decreased growth rate, reduced urine volume, and decreased activity, were noted in the pyridoxine-deficient group.

*Niacin excretion on diets with varying tryptophane content*

Twenty weanling rats, weighing 26–61 gm, were distributed among 4 large cages. The rats were all fed a basal ration containing 15% casein, and 0.2% L-cystine, with the composition otherwise as indicated earlier. The rats in 1 cage also received pyridoxine hydrochloride in the amount of 4 mg per 100 gm of ration; the basal ration contained no pyridoxine.

After 19 days on these rations, the rats were transferred to metabolism cages and the urine was collected during a 3-day period. On the twenty-second experimental day, the pyridoxine-deficient rats were divided into 3 groups. The first (— +) was given rations containing pyridoxine. The second (— —) was continued on the deficient diet. The third group (— + AL) was fed the ration with added pyridoxine ad libitum. The rats which had heretofore received pyridoxine (group + +) continued on the same ration, but their food intake was restricted to that of the first group (— +).

During the first period on this regime, the diet contained 15% casein (twenty-second to twenty-fifth day). L-tryptophane was then added in the proportion of 200 mg per 100 gm of diet (twenty-sixth to twenty-eighth day). In the third period (twenty-ninth to thirty-second day), the diet contained 30% casein, without added tryptophane. There followed periods on 30% zein (thirty-third to thirty-sixth day), and zein with added tryptophane. After 42 days, the animals in groups — + and + + were returned to the original 15% casein diet, and the series of variations in tryptophane and protein intake were repeated with some modifications. The results of the entire series are presented in tables 2 and 3. The figures for total tryptophane intake were computed on the basis of a

TABLE 3  
*Food intake and niacin excretion of rats fed diets varying in pyridoxine, protein and tryptophane content. Average values for 5 rats in each group.*

	EXPERIMENTAL PERIOD:										
	1	2	3	4	5	6	7	8	9	10	11
DAYS:	20-22	23-25	26-28	29-32	33-36	37-39	40-42	43-46	47-48	49-51	52-53
Proteins and amino acids fed	15	15	15	30	..	..	.	15	15		3.8
	.	..	.	..	30	30	30	.		30	23.0
			200	..	..	135	335	..	200	335	305
Casein, %											
Zein, %											
L-tryptophane, mg %											
Food intake, gm/rat/day	7.2	4.3	7.5	5.0	5.0	5.0	5.0	12.5	10.0	7.2	10.0
Total tryptophane intake, mg/rat/day	14.1	17.0	29.2	3.0	9.8	19.8	24.4	39.5	37.8	39.9	
Niacin excreted, µg/rat/day	14.6	19.3	50.0	42.5	14.6	29.3	29.3	15.6	28.4	90.0	64.0
N <sup>1</sup> -methylnicotinamide excreted, µg/rat/day	13.9	18.5	212.5	29.1	18.7	28.1	50.5	6.3	163.0	63.6	46.6
Food intake, gm/rat/day	7.2	4.3	7.5	5.0	5.0	5.0	5.0	12.1	10.0	7.4	10.0
Total tryptophane intake, mg/rat/day	14.1	17.0	29.2	3.0	9.8	19.8	24.4	39.5	37.8	39.9	
Niacin excreted, µg/rat/day	15.0	19.7	26.6	22.0	15.6	16.3	25.3	15.3	30.1	96.0	62.0
N <sup>1</sup> -methylnicotinamide excreted, µg/rat/day	26.3	20.8	126.2	37.4	20.0	51.3	63.5	14.5	91.7	86.6	26.1

<sup>1</sup> Group ++ fed pyridoxine throughout. Food intake restricted to that of group —+.

<sup>2</sup> Group —+ pyridoxine-deficient from first to twenty-second day, fed pyridoxine thereafter.

tryptophane content for casein of 1.3% (Greenhut et al., '46) and for zein, 0.2% (Schmidt, '38).

A number of important points appear in these tables. The most striking is probably the contrast between experimental periods 3 and 4. The excretion of niacin and methylnicotinamide was increased in all cases by the addition of L-tryptophane to the diet (period 3). When, however, the same amount

TABLE 3

*Food intake, and niacin excretion of rats fed diets varying in pyridoxine, protein and tryptophane content. Average values for 5 rats in each group.*

EXPERIMENTAL PERIOD:		1	2	3	4	5	6	7
DAYS:		20-22	23-25	26-28	29-32	33-36	37-39	40-42
Proteins	Casein, %	15	15	15	30	..	..	.
and amino	Zein, %	.	..	..	..	30	30	30
acids fed	L-tryptophane, mg %	.		200	.	..	135	335
Group								
— — <sup>1</sup>	Food intake, gm/rat/day		5.0	4.0	4.8	3.7	4.5	4.7
	Total tryptophane intake, mg/rat/day		9.8	15.8	18.7	2.2	9.2	18.6
	Niacin excreted, µg/rat/day	15.0	10.6	12.5	10.3	11.5	12.0	15.4
	N <sup>1</sup> -methylnicotinamide excreted, µg/rat/day	26.3	17.1	25.0	15.7	13.0	29.9	64.3
— + <sup>2</sup>	Food intake, gm/rat/day		8.0	8.0	10.0	5.2	6.7	6.1
AL	Total tryptophane intake, mg/rat/day		15.6	31.6	39.0	3.1	13.0	24.2
	Niacin excreted, µg/rat/day	15.0	9.9	36.6	26.0	18.6	21.8	42.5
	N <sup>1</sup> -methylnicotinamide excreted, µg/rat/day	26.3	5.3	112.3	62.9	41.4	26.6	67.6

<sup>1</sup> Pyridoxine-deficient throughout.

<sup>2</sup> Pyridoxine-deficient first to twenty-second day. Fed pyridoxine-containing diets ad libitum thereafter.

of tryptophane was added to the diet by doubling the casein content, the excretion of niacin and methylnicotinamide fell off very markedly. Evidently tryptophane, combined as casein, is not as effective in stimulating niacin synthesis as is free tryptophane. Moreover, in most instances, the addition of tryptophane to a diet containing zein has a less striking effect than similar additions to a casein diet.

In most cases, the response to added tryptophane appears as an increase in excretion of N<sup>1</sup>-methylnicotinamide, not niacin. This is particularly evident in the animals fed diets lacking in pyridoxine (group — —, table 3). Here, the niacin excretion remained at the same low level throughout the experiment. Such small increases as were observed after tryptophane were entirely confined to the methylated fraction. In the groups fed diets lacking in pyridoxine during the preliminary period, and then supplemented with pyridoxine later (group — +, table 2), the level of excretion was higher and the response to added tryptophane greater; here again, however, the response was most evident in the methylated fraction. The only exception appeared in the last experimental periods (10 and 11, table 2), where a definite increase in niacin excretion was evident. In animals fed pyridoxine throughout (group ++, table 2), the effect of added tryptophane was evident in both fractions, but was greatest in the methylated niacin. Again, the last experimental periods (10 and 11) constituted an exception; there niacin excretion increased, with a decrease in excretion of N<sup>1</sup>-methylnicotinamide.

#### DISCUSSION

There is no immediate explanation for all of the phenomena observed here. The relation of pyridoxine deficiency to niacin synthesis is a remarkable one; as Schweigert and Pearson ('47) have shown, animals on a diet lacking pyridoxine excrete less niacin after tryptophane feeding than animals fed pyridoxine. However, Rosen, Huff and Perlzweig ('47) report that addition of pyridoxine to a diet deficient in this



vitamin does not wholly restore the increased niacin excretion in response to tryptophane. Our results confirm both of these reports. The most obvious explanation for these phenomena would be that of synthesis by intestinal micro-organisms. In pyridoxine deficiency these symbionts would die out, and not be readily replaced on restoration of pyridoxine to the diet. However, the fact that increased excretion of niacin is observed after injection of tryptophane (Schweigert and Pearson, '47) suggests that the tissues of the rat are able to carry out the transformation. We are now investigating the effects of pyridoxine deficiency on the intestinal flora.

The facts that tryptophane, fed as casein, has little or no effect on niacin excretion, and that L-tryptophane, fed with zein, is less effective than is the same amino acid fed with casein, suggest that amino acids other than tryptophane are concerned in the formation of niacin. Krehl and coworkers ('46) have suggested that the poor growth of rats on corn diets low in niacin is the result of an amino acid antagonism in which glycine is an important factor. Amino acid antagonism has been reported frequently in the microbiological literature since Gordon and M'Leod ('26) showed inhibitory effects of several amino acids on growth of bacteria in protein digests. Sullivan, Hess and Sebrell ('32) and later workers have reported similar phenomena in animals.

Beadle, in a personal communication, has reported that, in *Neurospora*, tryptophane stimulates niacin synthesis, but is antagonized by leucine. This offers a possible explanation for the fact that tryptophane is less effective in the rat on a zein diet than on a diet containing casein; zein contains a much larger amount of leucine than does casein. Krehl et al. ('46) reported no inhibition in the growth of rats when leucine (1%) was added to a diet containing 9% of casein; no data were reported on niacin excretion. We are now examining the effect of leucine and other amino acids on the niacin excretion in response to tryptophane.

# SUMMARY

1. Addition of pyridoxine to a ration lacking this vitamin does not increase the excretion of niacin or N<sup>1</sup>-methylnicotinamide by the rat.

2. Rats fed rations deficient in pyridoxine do not excrete less niacin or N<sup>1</sup>-methylnicotinamide than rats fed rations adequately supplemented with this vitamin.

3. Addition of L-tryptophane to an adequate ration results in a marked increase in the excretion of both niacin and N<sup>1</sup>-methylnicotinamide.

4. Addition of L-tryptophane to a diet deficient in pyridoxine results only in a very small increase in excretion of N<sup>1</sup>-methylnicotinamide; niacin excretion is unchanged.

5. When rats are maintained on a diet deficient in pyridoxine for 22 days, and the diet is subsequently supplemented with pyridoxine, the increase in excretion of niacin after tryptophane is small. The excretion of N<sup>1</sup>-methylnicotinamide, however, increases greatly.

6. The increase in excretion of niacin and its methylated amide is greater when tryptophane is fed in a diet containing casein than in a diet containing zein as the protein.

7. Little or no increase in niacin or N<sup>1</sup>-methylnicotinamide is observed when the tryptophane intake is increased by feeding a high-protein diet.

8. The significance of these results is discussed. It is concluded that pyridoxine is concerned in the action of tryptophane in increasing niacin excretion, and that other amino acids are also involved, perhaps as antagonists to tryptophane.

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# MINERAL METABOLISM STUDIES IN DAIRY CATTLE

## II. EFFECT OF CALCIUM AND MANGANESE AND OTHER TRACE ELEMENTS ON THE METABOLISM OF LIPIDS DURING EARLY LACTATION <sup>1</sup>

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The literature regarding the interrelationships of mineral and fat metabolism has emphasized for the most part the effect of lipids on Ca and P metabolism with the greatest emphasis being placed on the antirachitic effect of lipids.

Holt et al. ('20, '23) found that the utilization of Ca by infants was augmented by fat in the diet, and Hickmans ('24) essentially verified their results. An increased absorption of Ca and P from the intestines of rats was observed by Boyd, Crum and Lyman ('32) when fat was added to the diet.

Holt, Tidwell and Kirk ('33), studying the effect of mineral intake upon fat retention in infants, found that an inverse relationship existed between the level of mineral intake and the portion of fat intake which was retained, with fat retentions of 81.4 and 94.8% for "high ash" and "low ash" diets, respectively. In a study of the feces from normal breast-fed and bottle-fed infants, Nielsen ('43) found that the total fat content of the dry feces was nearly twice as high for

<sup>1</sup> Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers University, Department of Dairy Industry. This research was supported by an appropriation from the Limestone Products Corporation of America, Newton, New Jersey.

bottle-fed infants while the total solids content was the same for both groups. He also noted that, in the breast-fed infants, fat was excreted chiefly in the form of free fatty acids and neutral fat and, to a considerably lesser degree, as soap, whereas in the bottle-fed infants the soap fraction in the dry feces averaged 78% of the total fat. The ash and CaO values of the dry feces were 8.94 and 1.86%, respectively, for the breast-fed infants and 23.45 and 9.50%, respectively, for the bottle-fed infants.

Williams et al. ('43), studying the fat partition of 19 healthy children from 4 to 12 years of age, found that excretion of free fatty acids, neutral fat and unsaponifiable material was approximately equal for all subjects but that soap excretion was significantly higher from children with larger fat intakes. For the group of children as a whole, soaps comprised approximately 50% of the total fecal fat; unsaponifiable fat represented 30%, and the remainder was almost equally divided between free fatty acid and neutral fat.

#### EXPERIMENTAL

The data reported in this paper were derived from the mineral balance trials given in paper I of this series (Reid et al., '47). Three weekly balance trials were conducted during the first 5 months of lactation at regular intervals with each of 8 Holstein and 4 Guernsey cows, comprising 3 collection and 4 feed groups.

The general feeding plan provided 1 pound of timothy-clover hay and 3.5 pounds of corn silage daily per 100 pounds body weight and a concentrate mixture, containing approximately 5% ether extractive material, in sufficient quantity to satisfy the Morrison ('40) feeding standard for maintenance and production plus 10%. Group I received the basal concentrate mixture; group II, basal concentrate mixture plus 3% of  $\text{CaCO}_3$  (c.p.); group III, basal concentrate mixture plus 3% of a mixture of  $\text{CaCO}_3$  (c.p.) and  $\text{MnSO}_4$  (c.p.) (proportions of Ca and Mn same as for group IV); group IV, basal

concentrate mixture plus 3% of a trace-element-fortified Ca supplement.<sup>2</sup>

The official method of the Association of Official Agricultural Chemists ('40) was used to determine the ether extractive content of the concentrate mixtures, hay, silage and feces.

The lipid fractionation was performed by the method of Forbes and Atkinson ('43) with modifications (Ward and Reid, '47). The method employs acid hydrolysis,  $\text{CHCl}_3$  as the lipid solvent, an artificial zeolite as the phospholipid adsorbent and dichromate oxidation as a means of lipid estimation. Neutral fat and sterols, free fatty acids and soaps, phospholipids and the total lipids were determined.

### RESULTS

The results obtained from the ether extraction and lipid fractionation analyses of the concentrate mixtures, hay, silage and feces were used to calculate the percentage of the intake of each fraction which was excreted in the feces. The loss percentages and average daily calcium intakes, their means, the standard errors of the means and the statistical significance of the differences between the mean lipid losses are presented in table 1. The proportion of ingested ether extractives lost in the feces of animals not receiving supplemental Ca (group I) was highly significantly greater than in that of animals receiving rations supplemented with Ca (groups II, III and IV). Although there was no significant difference among the means of the total lipid losses for the various groups, a significantly greater proportion of the ingested neutral fat and sterols was excreted in the feces of animals not receiving supplemental Ca (group I) than was lost by animals receiving increased quantities of this element (groups II, III and IV). The greater loss of neutral fat and sterols from group I animals was compensated to some extent by a greater retention of soaps and free fatty acids by these Ca unsupplemented cows.

<sup>2</sup> Mico, having the following percentage composition: calcium 33, magnesium 2, manganese 0.20, iron 0.20, iodine 0.045, copper 0.025, zinc 0.01 and cobalt 0.002.

Group III animals excreted a significantly greater proportion of the ingested soaps and free fatty acids than did those of groups I and II.

The results of this study point out the inadequacy of the ether extraction method as such for the measurement of total lipids in cow feces and particularly in the feces of cows receiving large quantities of Ca.

TABLE 1

*Mean percentages by fractions of ingested lipids excreted in the feces and the mean daily calcium intakes.*

GROUP NO.	DAILY CALCIUM INTAKE	FRACTION				
		Neutral fat and sterols <sup>1</sup>	Soaps and free fatty acids <sup>2</sup>	Phospho-lipids	Total lipids	Ether Extrac-tives <sup>3</sup>
	<i>gm</i>	<i>%</i>	<i>%</i>	<i>%</i>	<i>%</i>	<i>%</i>
I Mean <sup>4</sup>	47.97	44.68	24.34	50.55	32.88	23.57
St'd error	3.46	2.83	1.75	9.60	2.27	2.00
II Mean	99.23	34.74	26.49	34.72	29.77	12.17
St'd error	4.68	2.43	1.21	5.90	1.16	1.16
III Mean	97.17	36.08	30.55	33.51	32.42	14.76
St'd error	6.20	2.36	1.58	4.71	1.68	0.85
IV Mean	99.78	32.90	27.09	31.69	29.16	15.59
St'd error	3.22	2.32	2.02	6.26	2.07	1.17

<sup>1</sup> Mean differences are significant at the 5% level of probability (Fisher, '37) for groups I and II, I and III and at the 1% level of probability for groups I and IV.

<sup>2</sup> Mean differences are significant at the 5% level of probability for groups I and III and II and III.

<sup>3</sup> Mean differences are significant at the 1% level of probability for groups I and II, I and III, and I and IV.

<sup>4</sup> Each mean represents 9 values obtained from 3 animals, each on trial 3 times.

## DISCUSSION

It is notable that the mean loss of ingested total lipids via the feces was similar for the animals of all groups, whereas a highly significantly greater loss of the ingested ether extractives was found in the feces of the animals of group I, not receiving supplemental Ca. These data would indicate that

the utilization of total lipids by the lactating cow is not related to the Ca intake within the limits employed in this study; but, upon a comparison of the data dealing with ether extractives, it would follow that the extraction of some lipid materials by di-ethyl ether is precluded in a manner in some way related to the quantity of Ca ingested by the animal. Since soaps are not soluble in di-ethyl ether it is quite probable that they make up a large portion of the lipid material not accounted for in the feces by the ether extraction procedure. Because of this shortcoming of the usual ether extraction method, it is evident that the apparent digestibility of crude fat would be unjustifiably elevated when cows receive high Ca rations, as is shown by the data for ether extractives in table 1.

Since the total lipid excretion was of similar magnitude in all groups and since group I excreted the largest proportion of neutral fat and sterols and the smallest proportion of soaps and free fatty acids, it would seem to be indicated that the increased Ca intake was largely responsible for the sparing of neutral fat at the expense of soaps and free fatty acids.

Kon and Booth ('33) showed that fat had a sparing action on Ca as shown by its antirachitic effect. In view of the data presented here, it would seem that the statement can be reversed in the case of the lactating cow, i.e., that Ca has a sparing action on the neutral fat and sterol fraction, while the soaps and free fatty acids are simultaneously expended. In contrast to this, Holt et al. ('33) found that the lipid loss in the feces of infants was directly proportional to the ash content (largely made up of Ca) of the diet.

It is possible that other mineral elements in the diet affect the lipid retention but, under the conditions of this experiment and in the amounts of Mn, I, Fe, Cu, Zn and Co supplemented, only Mn when added singly to the calcium had a significant effect, and that was qualitative rather than quantitative.



## SUMMARY

A study was made of the effects of Ca, Mn, and other mineral element supplementation upon the metabolism of lipids during the first 5 months of lactation of 8 Holstein and 4 Guernsey cows.

All groups excreted similar proportions of the total lipid intake, as measured by acid hydrolysis and  $\text{CHCl}_3$  extraction, regardless of the mineral supplement received. Therefore the observed group differences relative to the smaller proportion of ether extractives lost in the feces of animals receiving supplemental Ca were attributed to an increased formation of soaps which were not measured in the feces of these animals because of the insolubility of soap in di-ethyl ether, and not to a more efficient lipid use. Although the ingestion of Ca tended to spare neutral fat and sterols, a corresponding increase was observed in the excretion of soaps and free fatty acids, resulting in a qualitative rather than a quantitative effect.

These data would indicate that the estimation of crude fat by the commonly employed ether extraction procedure is likely to be misleading in experiments involving the analysis of dairy cow feces.

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# THE BIOLOGICAL VALUE OF THE PROTEIN OF FIELD PEA PRODUCTS WITH A COMPARISON OF SEVERAL METHODS USED FOR THIS DETERMINATION <sup>1</sup>

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Field peas (*Pisum sativum*) have been extensively employed as a source of protein in famine areas in Europe and Asia as well as in low cost dietaries in the United States. Cull peas provide a protein concentrate of high value for animal feeding. Woods et al. ('43) and Russell et al. ('46) working with rats, and Peterson and Lampman ('44) using chickens, demonstrated that a low methionine content was a limiting factor when peas were used as the sole source of protein in growth experiments. Lehrer et al. ('47) in growth trials with rats have shown that meat supplements overcome this deficiency.

An examination of the literature points to some controversies regarding adequate methods for measuring biological value of proteins. Mitchell ('44) showed that the ratio of body weight gain to protein eaten may be changed by increasing food intake. Thus, a large experimental error may result in this ratio when ad libitum feeding is employed, tending to favor the efficiency of the better protein which is usually consumed in larger amounts. Barnes and Bosshardt ('46)

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concluded that pair-feeding gives a low value for the better protein since, if restriction is severe, some of it may be wasted for fuel. These workers cite evidence to prove that for several different proteins when there is maximal utilization, the amount absorbed is the same. Therefore, by ad libitum feeding, protein intake may be equalized and the greatest utilization obtained.

The purpose of the experiments in Part I of this paper was to determine (a) the biological value of the protein of various pea products, both raw and cooked, and (b) the effect of supplementation with various cereals. The purpose of the work reported in Part II was to make a comparison of 3 methods used for the estimation of biological value, namely, protein efficiency and retention in pair-fed versus ad libitum trials and a metabolism study using diets consisting of 10% casein and raw pea protein.

PART I. THE BIOLOGICAL VALUE OF VARIOUS PEA PRODUCTS  
—THE EFFECT OF SUPPLEMENTATION AND HEAT  
ON PROTEIN EFFICIENCY

*Experimental*

The germ, the sprouts and the entire seed, both from immature and mature plants of the Alaska field pea were studied. The germ resulted from the splitting process and constituted approximately 40% of the mixture, the rest consisting of fragments of the cotyledon. Peas were allowed to germinate until the sprouts were 2 to 3 inches in length, then blanched and dehydrated. Immature peas were harvested at the canning or freezing stage, blanched and dehydrated. The mature seed was used as harvested. Canned peas were soaked overnight, parboiled, packed in no. 2½ cans, sealed and autoclaved for 40 minutes at 10 pounds pressure (240°F.), then dehydrated. Baked peas were prepared by soaking overnight, parboiling for 15 minutes, and then baking for 2 hours at 375°F.; the product was then dehydrated. All dried products were ground and then analyzed for moisture and nitrogen.

The quantity of each required to furnish a protein level ( $N \times 6.25$ ) of 10 or 15% was then used in making up the diets (table 1). Due to their high methionine content, cereals (wheat, corn and barley) were used as supplements in some of the diets.

Each rat received as a supplement 3 times a week a vitamin solution containing thiamine hydrochloride 50  $\mu$ g, riboflavin 50  $\mu$ g, pyridoxine 50  $\mu$ g, calcium pantothenate 250  $\mu$ g, nicotinic acid 580  $\mu$ g, inositol 130  $\mu$ g, choline hydrochloride 20 mg,

TABLE 1  
*Percentage composition of diets.*

INGREDIENTS	WHOLE PEA	CASEIN	PEA SPROUT	PEA GERM	PEA <sup>1</sup> WHEAT	IM- MATURE PEA	PEA BAR- LEY	PEA CORN	PEA <sup>1</sup> WHEAT
Whole pea	44.6	...	...	...	30.0	...	26.6	26.6	22.3
Casein	...	11.0	...	...	...	...	...	...	...
Pea sprouts	...	...	41.3	...	...	...	...	...	...
Pea germ	...	...	...	31.7	...	...	...	...	...
Whole wheat	...	...	...	...	37.0	...	...	...	46.3
Immature pea	...	...	...	...	...	36.2	...	...	...
Pearl barley	...	...	...	...	...	...	38.3	...	...
Yellow corn	...	...	...	...	...	...	...	40.0	...
Sugar or starch <sup>2</sup>	42.9	76.5	46.2	55.8	20.5	51.3	22.6	20.9	18.9
Hydrogenated fat	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Hubbell salts <sup>3</sup>	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5

<sup>1</sup> This combination was tested twice, one containing less pea than the other.

<sup>2</sup> Starch was substituted for sugar in diets used in experiments 3 and 4 due to wartime shortage.

<sup>3</sup> Hubbell et al. ('37).

p-aminobenzoic acid 250  $\mu$ g, vitamin A 330 I.U., vitamin D 33 I.U., and mixed tocopherols 2 mg.

Animals were paired as to sex and size, and placed on experiment when they weighed 40 to 60 gm. They were housed in individual cages and weighed 3 times weekly. The trials in experiments 1 and 2 and the group on casein in experiment 3 were run on animals pair-fed for equal food consumption; the food intake of animals on each diet was regulated to that of the one on the raw pea diet.

*Results and discussion*

Results of this part of the study are summarized in tables 2 and 3. When casein and pea protein were compared on the basis of protein efficiency (gain per gm of protein eaten), casein proved to be approximately 50% better. The germ appeared to be slightly but not significantly superior in quality to the whole pea. Both sprouting and harvesting at the immature stage significantly lowered protein efficiency. This is contrary to the work on soybeans of Everson et al. ('44), who found that the germinated and raw, immature beans were distinctly superior to the mature ones.

The addition of cereals (corn, wheat and barley) materially improved the efficiency of the pea protein. The 3 cereals when substituted at a 5% protein level gave quite comparable results, all of which showed significant improvement over the pea protein alone. Apparently supplementation below the 5% level is not adequate as shown by a lower value where wheat contributed only one-third of the protein. The improvement due to cereal protein can no doubt be attributed to the high methionine content of these grains, thus compensating somewhat for this amino acid deficiency in peas.

Woods et al. ('43), in their work on the biological value of the field pea, secured an average protein efficiency of 1.0, and Everson and Heckert ('44) obtained a figure of 0.76 for green peas and 0.63 for the yellow variety. Values reported by these workers are somewhat lower than the figure in this study but the difference in value between casein and raw pea was quite comparable in the 3 investigations. Comparison of the results in table 2 with those of previous reports is valid since growing rats of approximately the same weight were used in all studies.

Raising the level of pea protein to 15% produced significantly greater gains but did not alter the efficiency. These findings are in agreement with those of Woods et al. ('43) where they compared raw pea protein at the 10 and 20% level. However, Jones and Widness ('46) secured a lowered

TABLE 2

*Growth response of rats on various diets used to determine biological value of pea protein.*

DIETS	NO. OF ANIMALS	AVERAGE TOTAL GAIN	AVERAGE GAIN PER GRAM OF PROTEIN	AVERAGE FOOD REQUIRED PER GRAM OF GAIN	AVERAGE FOOD EATEN DAILY PER RAT
		gm	gm	gm	gm
Experiment <sup>1</sup> no. 1 (pair-fed)					
10% raw pea	10	66.5 ± 5.0 <sup>2</sup>	1.38 ± 0.06 <sup>2</sup>	6.58 ± 0.30 <sup>2</sup>	7.6
10% pea sprout	8	51.5 ± 2.0	1.23 ± 0.03	7.91 ± 0.16	7.3
10% raw pea germ	8	71.0 ± 4.4	1.58 ± 0.09	6.12 ± 0.33	7.7
3½% whole wheat } 6¾% raw pea }	10	79.8 ± 5.8	1.58 ± 0.07	5.51 ± 0.36	7.6
Experiment <sup>1</sup> no. 2 (pair-fed)					
10% raw pea	10	52.5 ± 2.2	1.47 ± 0.06	6.60 ± 0.29	8.4
5% raw pea } 5% whole yellow corn }	10	70.7 ± 2.6	1.98 ± 0.09	4.91 ± 0.22	8.2
5% raw pea } 5% pearl barley }	10	71.1 ± 2.3	1.78 ± 0.06	4.91 ± 0.16	8.3
5% raw pea } 5% whole wheat }	10	66.5 ± 2.1	1.80 ± 0.05	5.11 ± 0.14	8.1
10% immature pea	10	42.5 ± 2.0	1.16 ± 0.05	8.09 ± 0.40	8.1
Experiment <sup>1</sup> no. 3 (pair-fed)					
10% casein	8	45.9 ± 2.9	1.97 ± 0.08	5.49 ± 0.68	5.9
10% raw pea	8	33.8 ± 1.5	1.36 ± 0.09	7.19 ± 0.31	5.8
10% baked pea	8	35.4 ± 2.3	1.06 ± 0.05	9.77 ± 0.49	8.0
10% canned pea	7	17.5 ± 1.5	0.71 ± 0.05	11.38 ± 0.95	6.0
Experiment <sup>1</sup> no. 4 (pair-fed)					
10% casein	9	70.1 ± 3.2	1.82 ± 0.08	5.60 ± 0.24	9.3
10% raw pea	9	49.1 ± 1.6	1.23 ± 0.06	8.18 ± 0.40	9.5
Ad libitum feeding					
10% casein	9	87.4 ± 4.2	2.05 ± 0.07	4.93 ± 0.15	10.0
10% raw pea	9	53.0 ± 2.2	1.34 ± 0.06	7.48 ± 0.31	9.3
15% raw pea	10	102.7 ± 3.6	1.33 ± 0.03	5.02 ± 0.13	12.2

<sup>1</sup> Duration of experiment 1 was 8 weeks; experiments 2, 3 and 4 ran for 6 weeks.

<sup>2</sup> Standard error of single observations.



TABLE 3

*Statistical comparisons of growth on various diets.*

DIETS COMPARED	GRAMS GAINED		GRAMS GAINED PER GRAM PROTEIN	
	Mean Difference	t <sup>1</sup>	Mean Difference	t <sup>1</sup>
Experiment no. 1 (pair-fed)				
10% raw pea vs. 10% pea sprout	15.0 ± 5.4 <sup>2</sup>	2.77 <sup>2</sup>	0.15 ± 0.07 <sup>2</sup>	2.14 <sup>2</sup>
10% raw pea vs. 10% pea germ	4.5 ± 6.6	0.70	0.20 ± 0.10	2.00
10% raw pea vs. 6 $\frac{2}{3}$ % raw pea and 3 $\frac{1}{3}$ % whole wheat	13.3 ± 7.6	1.75	0.20 ± 0.09	2.22 <sup>2</sup>
Experiment no. 2 (pair-fed)				
10% raw pea vs. 5% raw pea-5% corn	17.5 ± 3.4	5.15 <sup>4</sup>	0.51 ± 0.11	4.63 <sup>2</sup>
10% raw pea vs. 5% raw pea-5% barley	18.6 ± 3.2	5.81 <sup>4</sup>	0.31 ± 0.08	3.87 <sup>2</sup>
10% raw pea vs. 5% raw pea-5% wheat	14.0 ± 3.0	4.67 <sup>4</sup>	0.33 ± 0.08	4.11 <sup>2</sup>
10% raw pea vs. 10% immature pea	10.0 ± 2.9	3.45 <sup>4</sup>	0.31 ± 0.08	3.87 <sup>2</sup>
Experiment no. 3 (pair-fed)				
10% raw pea vs. 10% canned pea	16.3 ± 2.1	7.76 <sup>4</sup>	0.65 ± 0.10	6.50 <sup>4</sup>
10% raw pea vs. 10% baked pea	1.6 ± 2.1	0.76	0.30 ± 0.10	3.00 <sup>4</sup>
Experiment no. 4				
10% casein vs. 10% raw pea pair-fed	21.0 ± 3.5	6.00 <sup>4</sup>	0.59 ± 0.10	5.90 <sup>4</sup>
10% casein vs. 10% raw pea ad libitum fed	34.4 ± 4.7	7.32 <sup>4</sup>	0.71 ± 0.09	7.89 <sup>4</sup>
10% casein pair-fed vs. 10% casein ad libitum fed	17.3 ± 5.2	3.33 <sup>4</sup>	0.23 ± 0.13	1.77
10% raw pea vs. 15% raw pea	53.6 ± 3.9	13.73 <sup>4</sup>	0.10 ± 0.07	1.43

<sup>1</sup> Calculated from Fisher's formula (Snedecor, '38).<sup>2</sup> Standard error.<sup>3</sup> Denotes significance at the 5% level.<sup>4</sup> Denotes significance at the 1% level.

efficiency in tests of a series of proteins of vegetable origin (wheat, corn germ, soybean, cottonseed and peanut flour) with increasing protein content of the diets, comparisons being made at a 10, 15 and 17.5% level.

Baking lowered the efficiency of the protein significantly, despite the fact that consumption of this diet was greater than that of the raw pea. Woods et al. ('43) found that supplementation of autoclaved pea protein with 0.5% cystine restored the efficiency to that of the raw pea. Evans and St. John ('47) report that losses amounting to 24% of the cystine in peas result from autoclaving for 60 minutes at 130°C., whereas no change takes place in the methionine content. Since the rat's requirement for sulfur-containing amino acids is high (Cox et al., '47) and the amount in peas is low, losses would quickly be registered in a decreased growth rate. This deficiency also causes the loss of guard hairs and the development of a soft, short fur after the animal has been on experiment for several weeks.

## PART II. A COMPARISON OF METHODS FOR DETERMINING BIOLOGICAL VALUE OF PROTEINS

### *Experimental*

In experiment 4 a comparison was made of the pair-fed versus ad libitum methods using 10% raw pea and 10% casein diets. The trials were conducted as were those in experiments 1, 2 and 3 in Part I, with the exception that 1 group of animals on the casein diet was fed ad libitum. At the conclusion of the experiment, animals were killed, the gastrointestinal tract removed and the ground carcass analyzed for moisture and protein. Protein storage was obtained by subtracting the protein content of a group of control animals. Nitrogen determinations were run by the macro Kjeldahl method.

The method of Mitchell ('24) and Mitchell and Carman ('26) was used for the metabolism study. The 10% casein and 10% raw pea diets were employed during the test period. The

standardizing diet contained 4% whole egg protein and sufficient additional fat to make the total 10% correspond to the fat content of the test diets. All diets contained 2% agar for bulk. Test periods were 10 days in duration and collections were made for the last 6 days. Three periods were run on the standardizing diet, 1 each for the test proteins.

### *Results and discussion*

In order to compare the 2 methods of feeding (pair and ad libitum) the growth rate of animals on a 10% casein diet was used (tables 2 and 3). A significant difference was observed in their weight gains but not in protein efficiency.

TABLE 4

*Comparison of methods for determination of biological value.  
Pair-feeding versus ad libitum feeding.*

	NO. OF ANI- MALS	DIET	TOTAL PROTEIN STORED	PROTEIN STORED PER GRAM OF PROTEIN EATEN	MOISTURE
			gm	gm	%
Pair-fed	9	10% casein	$15.00 \pm 0.70^1$	$0.39 \pm 0.02^1$	$62.75 \pm 1.06^1$
	9	10% raw pea	$9.37 \pm 0.48$	$0.25 \pm 0.01$	$63.30 \pm 0.63$
			$t^2 = 6.62^2$	$t^2 = 7.00^3$	
Ad libitum	9	10% casein	$17.64 \pm 1.18$	$0.41 \pm 0.02$	$64.38 \pm 0.57$
	9	10% raw pea	$9.72 \pm 0.47$	$0.25 \pm 0.01$	$64.44 \pm 0.45$
			$t^2 = 6.71^2$	$t^2 = 8.00^3$	

<sup>1</sup> Standard error of single observations.

<sup>2</sup> Calculated from Fisher's formula (Snedecor, '38).

<sup>3</sup> Denotes significance at the 1% level.

Ad libitum feeding did not result in more protein storage per gram of protein fed (0.41 for this group contrasted with 0.39 per gm for those pair-fed, table 4). Since the 2 methods of feeding appear to be comparable, the selection of the one to be used would be a matter of choice. However, if a complete protein such as that of whole egg had been employed in lieu of casein, ad libitum feeding would no doubt have resulted in a significant increase in both protein efficiency and retention.

Biological value expressed either as protein efficiency (table 2) or protein stored per gm eaten (table 4) resulted in about the same difference (approximately 55%) between the casein and raw pea protein.

The biological value of casein at a 10% level determined in a metabolism study proved to be 65.6%, that for peas 57.3%, a difference of 8%. The coefficient of true digestibility for casein from this study was 98.6% and that for peas 91.6%, a difference of 7%. It is doubtful whether the lower digestibility of the pea protein completely explains the lower biological value, as Woods et al. ('43) were able to elevate the protein efficiency of a 10% pea diet above that of casein by the addition of 0.3% methionine.

#### SUMMARY

1. The protein of raw Alaska field peas is inferior to that of casein when both are fed at the 10% level. Of the pea products studied as sources of protein the germ proved to be slightly but not significantly more efficient, whereas both the sprout and immature pea were significantly lower in value than the raw pea.

2. Supplementation of at least half of the raw pea protein with cereal grains materially improved the biological value presumably because this increased the methionine content.

3. Canning has a very harmful effect on the pea protein probably because the cystine content is lowered by this process. Baking, too, lowers the value significantly.

4. Three methods of determining the biological value of proteins have been compared: protein retention, protein efficiency and a metabolism study. Biological value expressed either as protein efficiency or protein stored per gram eaten resulted in about the same difference (approximately 55%) between the casein or pea protein.

5. The biological value of casein at a 10% level determined in a metabolism study proved to be 65.6%, that for peas, 57.3%, a difference of 8%. The coefficients of true digestibility

for casein and peas from this study were 98.6% and 91.6%, respectively, a difference of 7%.

6. A comparison of 2 groups of animals (pair and ad libitum fed) on a 10% casein diet showed a significant difference in weight gains but not in protein efficiency or protein stored per gram of protein eaten.

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# THE EFFECTS OF B VITAMINS, LIVER AND YEAST ON ATABRINE TOXICITY IN THE RAT<sup>1</sup>

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Available data indicate that in addition to the major nutrients, there are substances present in our diet which may be required in increased amounts during conditions of stress. Such factors are apparently dispensable under normal conditions, or their requirements are so small that they may readily be met by amounts present in the diet or through the synthetic activity of the intestinal flora or the animals' own tissues. Certain drugs or other "stress factors" may, however, increase requirements for these substances to such an extent that deficiencies occur, manifested by retarded growth or tissue pathology, and preventable by the administration in appropriate amounts of the missing nutrient (Ershoff, '48). In the present communication data are presented indicating that atabrine is such a stress factor. Administration of this drug in toxic amounts to the young rat resulted in retardation of growth, alopecia, inhibition of ovarian development, granulocytosis and other pathological effects preventable, at least in part, by the administration of an unknown nutrient present in liver and yeast.

<sup>1</sup> The research which this paper reports was undertaken in cooperation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The views or conclusions contained in this report are those of the author. They are not to be construed as necessarily reflecting the views or indorsement of the War Department.



## PROCEDURE AND RESULTS

Four basal rations were employed in the present experiment: diets A, B, C and D. Diets A and B were purified rations containing the B complex factors in synthetic form only. Diets C and D were similar in composition but contained yeast or desiccated whole liver in addition to the synthetic vitamins. For the present experiment 64 female rats of the Long-Evans strain were selected at 21 to 23 days of age and with an average weight of 41.8 gm. The animals were kept in metal cages with raised screen bottoms to prevent access to feces, and were fed *ad libitum* the diets listed in table 1. Feeding was continued for 8 weeks or until death, whichever occurred sooner. During the eighth week electrocardiograms were taken of all rats. All animals were autopsied on the fifty-sixth day of feeding, ovarian and submaxillary weights determined, and sections of the ovaries, submaxillary glands, liver and heart prepared for microscopic study. Tissues were fixed in 10% formol and stained with hematoxylin and eosin.

Our findings indicate that the toxic effects of atabrine in the immature rat may be counteracted by dietary means. Pathological effects of atabrine were most pronounced in rats subsisting on the synthetic ration (diet A), and least evident in the animals on the liver-containing diet D. Atabrine-fed rats on diet A exhibited a marked retardation of growth (table 2), extensive alopecia, infantile ovaries and enlarged submaxillary glands (table 2), granulocytosis (table 3) and electrocardiographic abnormalities. Three of the 10 rats on this diet failed to survive the experimental period of 8 weeks; and of the remaining 7 animals only 2 gained weight after the second week of feeding. Eight rats in this series developed extensive alopecia during the first month of feeding although new hair had generally replaced the areas of alopecia by the eighth week. At autopsy ovaries appeared infantile both in weight and microscopic appearance. The most striking finding, however, was a marked hypertrophy of the submaxillary glands. These weighed 3 to 4 times more than those observed in normal

TABLE 1

*Composition of experimental diets without (—) and with (+) atabrine.<sup>1</sup>*

COMPONENTS OF BASAL MIXTURE	DIETS A <sub>1</sub> and A <sub>2</sub>	DIETS B <sub>1</sub> and B <sub>2</sub>	DIETS C <sub>1</sub> and C <sub>2</sub>	DIETS D <sub>1</sub> and D <sub>2</sub>
	— +	— +	— +	— +
Yeast <sup>2</sup>	0.0	0.0	10.0	0.0
Whole liver powder <sup>3</sup>	0.0	0.0	0.0	10.0
Casein, "vitamin-test" <sup>4</sup>	22.0	22.0	22.0	22.0
Sucrose	73.5	73.5	63.5	63.5
Salt mixture <sup>5</sup>	4.5	4.5	4.5	4.5

*Vitamin supplements*

Fat-soluble vitamins: for all diets, see footnote 6.

B vitamins: for diets A, C and D, to provide levels considerably in excess of minimum requirements, see footnote 7; for diet B, see footnote 8.

<sup>1</sup> Atabrine (quinacrine-HCl powder, Winthrop Chemical Co., New York) was incorporated in diets A<sub>2</sub>, B<sub>2</sub>, C<sub>2</sub> and D<sub>2</sub> at a level of 500 mg per kg of diet, replacing an equal amount of sucrose.

<sup>2</sup> Brewers' type yeast no. 200, Anheuser-Busch, Inc., St. Louis, Mo. Each gram, according to the manufacturer, contained the following vitamin potencies: thiamine 600 µg, riboflavin 75 µg, pyridoxine 100–125 µg, pantothenic acid 420–560 µg and nicotinic acid 350–500 µg.

<sup>3</sup> Whole dried liver powder, Armour and Co., Chicago, Ill.

<sup>4</sup> Vitamin-test casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

<sup>5</sup> Salt mixture no. 1 of Sure ('41).

<sup>6</sup> Each rat received 3 times weekly the following supplement: cottonseed oil (Wesson) 500 mg, alpha-tocopherol 1 mg, and a vitamin A–D concentrate containing 50 U.S.P. units of vitamin A and 5 U.S.P. units of vitamin D. The concentrate used was Nopco assaying 800,000 U.S.P. units of vitamin A and 80,000 U.S.P. units of vitamin D per gram.

<sup>7</sup> To each kilo of diets A, C and D were added the following synthetic vitamins: thiamine hydrochloride 72 mg, riboflavin 9 mg, pyridoxine hydrochloride 15 mg, calcium pantothenate 67.2 mg, nicotinic acid 60 mg, 2-methyl-1,4-naphthoquinone 5 mg and choline chloride 1.2 mg. The thiamine, riboflavin, pyridoxine, pantothenate and nicotinic acid were administered in amounts comparable to those provided by the brewers' type yeast no. 200 when fed at a level of 12% of the diet.

<sup>8</sup> To each kg of diet B were added thiamine hydrochloride 144 mg, riboflavin 18 mg, pyridoxine hydrochloride 30 mg, calcium pantothenate 134.4 mg, nicotinic acid 120 mg, p-aminobenzoic acid 600 mg, folic acid 10 mg, biotin 1 mg, 2-methyl-1,4-naphthoquinone 10 mg, choline chloride 1.2 gm and inositol 1.2 gm. Doubling the content of these vitamins in diet B (over A, C and D; see footnote 7) provided these factors in amounts comparable to those present in diet C.

animals of similar weight and were significantly heavier than those observed in the much larger atabrine-free controls (table 2). Histologically the increased size of the submaxillary glands appeared to be due almost entirely to hyperplasia and hypertrophy of mucous cells.<sup>2</sup>

Retardation of growth was similarly observed in atabrine-fed rats on diets B, C and D although growth was significantly greater in rats on these rations than in those on diet A. Atabrine-fed rats gained most weight on the liver-containing diet D, with growth somewhat less on diets containing yeast (diet C) or the additional B vitamins (diet B). The growth curves of 3 rats on the latter ration showed a plateau after the first few weeks of feeding; the remainder of the group, however, and all atabrine-fed rats on diets C and D gained weight consistently during the experimental period. With the exception of the 3 rats on diet B whose growth curves showed a plateau, alopecia was not observed in any of the atabrine-fed rats except those on diet A.

The ovaries appeared infantile both in weight and microscopic appearance in all atabrine-fed rats on diet A. They were somewhat larger in atabrine-fed rats on diets B and C but were still significantly smaller than those observed in atabrine-free controls. On diet D no significant difference in ovarian weight was observed between animals fed atabrine and those on similar rations with atabrine omitted; and histologically the ovaries of atabrine-fed rats in this group appeared normal in all respects. On atabrine-free rations ovarian weights did not differ significantly on any of the diets employed; and histologically, ovaries appeared normal in all groups (table 2).

Enlargement of the submaxillary glands was directly correlated with alopecia and failure to gain weight. Enlarged submaxillaries were only observed in atabrine-fed rats that lost fur and whose growth curves showed a plateau (8 rats on diet A; 3 on diet B), with glands apparently normal both in

<sup>2</sup> We are indebted to Professor E. M. Hall, Department of Pathology, University of Southern California Medical School, for examination of the histological material.

size and histological appearance in the remaining animals of the atabrine series. In no instance where atabrine-free rations were used were the submaxillary glands enlarged (table 2).

TABLE 2

*Effects of atabrine on growth and ovarian and submaxillary weight in the immature female rat.*

DIETARY GROUP	NUMBER OF ANIMALS	INITIAL BODY WEIGHT	GAIN IN BODY WT. OVER 8-WK. PERIOD	AVERAGE OVARIAN WT. <sup>1</sup>	AVERAGE SUBMAXIL-LARY WT. <sup>1</sup>	RATIO OF SUBMAXIL-LARY WT. TO BODY WT. $\times 10^{-3}$
		gm	gm	mg	mg	
Atabrine series						
A	10	41.7	33.9 $\pm$ 12.1 (7) <sup>2</sup>	12.4 $\pm$ 3.4	486.4 $\pm$ 41.6	6.43
B	10	41.9	87.4 $\pm$ 10.8 (9)	26.0 $\pm$ 3.5	418.6 $\pm$ 39.4	3.24
C	10	41.8	33.1 $\pm$ 5.1 (10)	30.4 $\pm$ 2.7	303.8 $\pm$ 10.8	2.15
D	10	41.8	118.2 $\pm$ 5.2 (10)	43.5 $\pm$ 2.9	317.8 $\pm$ 19.4	1.92
Atabrine-free controls						
A	6	41.7	146.4 $\pm$ 9.0 (6)	47.0 $\pm$ 2.4	374.8 $\pm$ 18.4	1.98
B	6	42.0	152.3 $\pm$ 7.8 (6)	49.1 $\pm$ 3.0	334.6 $\pm$ 16.8	1.72
C	6	41.5	146.8 $\pm$ 6.9 (6)	43.2 $\pm$ 3.2	317.3 $\pm$ 32.8	1.69
D	6	41.7	169.7 $\pm$ 9.3 (6)	44.8 $\pm$ 2.1	313.5 $\pm$ 14.8	1.49

<sup>1</sup> Including standard error of the mean calculated as follows:  $\sqrt{\sum d^2/n}/\sqrt{n}$  where "d" is the deviation from the mean and "n" is the number of observations.

<sup>2</sup> The values in parentheses indicate the number of animals which survived and on which averages are based.

Both the per cent and the total number of granulocytes per milliliter of blood were markedly increased in atabrine-fed rats fed diet A. During the sixth week of feeding total and differential white cell counts, hemoglobin determinations and total red cell counts were made on the tail blood of all sur-

viving rats. Differential counts were made on smears stained with Wright's stain, 100 cells on each of 2 slides being employed for each analysis. All blood counts were made in duplicate.

On the various diets tested no significant difference in total erythrocytes or hemoglobin levels was observed between animals fed atabrine and those on atabrine-free rations. Erythrocytes averaged 7.3 to 8.1 million per mm<sup>3</sup> of blood for the various groups (range 6.4-9.3), with hemoglobin averaging

TABLE 3  
*Effects of atabrine on the granulocyte count of the rat.*

DIETARY GROUP	NUMBER OF ANIMALS	TOTAL LEUCOCYTE COUNT		GRANULOCYTES	
		Average <sup>1</sup> per mm <sup>3</sup>	Range per mm <sup>3</sup>	% <sup>1</sup>	Total <sup>1</sup> per mm <sup>3</sup>
Atabrine series					
A	8	17,090 ± 1,540	10,200-24,800	43.9 ± 4.3	7,503 ± 735
B	10	15,900 ± 1,310	8,600-24,100	23.0 ± 1.7	3,657 ± 270
C	10	13,280 ± 1,650	9,200-20,400	24.3 ± 2.5	3,227 ± 332
D	10	12,270 ± 1,280	7,900-18,400	21.5 ± 2.3	2,638 ± 282
Atabrine-free controls					
A	6	13,150 ± 860	8,800-15,800	18.6 ± 2.6	2,446 ± 342
B	6	12,870 ± 1,040	7,800-18,100	17.4 ± 2.8	2,239 ± 360
C	6	14,180 ± 810	8,200-16,600	19.1 ± 1.6	2,708 ± 227
D	6	13,710 ± 930	10,600-15,600	24.2 ± 2.0	3,318 ± 274

<sup>1</sup> Including standard error of the mean calculated as follows:  $\sqrt{\sum d^2/n}/\sqrt{n}$  where "d" is the deviation from the mean and "n" is the number of observations.

15.4 to 16.1 mg/100 ml (range 14.6-17.2). Total leucocytes did not differ significantly for the various rations tested; a significant increase in per cent and total granulocytes was observed, however, in atabrine-fed rats on diet A. Such was not the case with atabrine-fed rats on other diets tested nor for animals on atabrine-free rations (table 3).

In agreement with earlier findings (Hegsted, McKibbin and Stare, '44) no consistent abnormalities were observed histologically in the liver and myocardium of atabrine-fed rats on the various diets employed, nor did these tissues differ sig-

nificantly from those of animals fed similar rations with atabrine omitted.<sup>3</sup> These findings are in contrast to those of Wright and Lillie ('43) and Siegel and Mushett ('44) who observed necrosis and a replacement fibrosis in the liver and myocardium of atabrine-fed rats. These differences may be due, at least in part, to the amount of atabrine fed, the composition of the diets employed or a strain difference in response to atabrine feeding.

Myocardial damage in atabrine-fed rats was indicated, however, by means of electrocardiographic tracings. Electrocardiograms were taken with a Cambridge-Hindle 2 galvanometer electrocardiograph (research type unit) of all surviving rats during the eighth week of feeding. Resistance was standardized for each animal, and the standard 3 leads were taken on unanesthetized rats at a paper speed of 100 cm per second.<sup>4</sup> A marked elevation of the ST segment (indicative of myocardial damage) was observed in the electrocardiographic tracings of 5 of the 7 atabrine-fed rats on diet A, and 5 of the 9 atabrine-fed rats on diet B.<sup>5</sup> Elevated ST segments did not occur in tracings obtained from rats fed diets C<sub>2</sub> or D<sub>2</sub> or from animals fed atabrine-free rations. Further abnormalities in the atabrine series consisted of a prolonged PR interval (indicative of delayed AV conduction) in animals fed diet D<sub>2</sub>. Six of the 10 rats on the latter ration had a PR interval of 0.05 seconds or longer in contrast to an approximate value of 0.04 seconds in virtually all rats fed other rations tested. With the exceptions of the above, electrocardiograms of atabrine-fed rats did not differ significantly

<sup>3</sup> See footnote 2, page 272.

<sup>4</sup> We wish to express our sincere appreciation to Dr. John C. Ruddock, for the examination and description of the electrocardiographic tracings. Dr. Ruddock is Clinical Professor of Medicine, University of Southern California Medical School, Chief of Medical Service and Head of the Department of Cardiology, St. Vincent's Hospital, Los Angeles, California.

<sup>5</sup> ST segments were elevated on all 3 leads in rats on diet B<sub>2</sub> and on leads II and III in those on diet A<sub>2</sub>. Slight elevations of the ST segment were occasionally noted in leads II and III in animals on all control rations and those on diets C<sub>2</sub> and D<sub>2</sub>. In no case, however, were they so pronounced as those in rats on diets A<sub>2</sub> or B<sub>2</sub>.

from those of the controls either in complexes or in ventricular rate.<sup>6</sup> No abnormalities were observed in the electrocardiograms of atabrine-fed rats on diet C.

In subsequent work experiments were conducted in an attempt to concentrate the factor or factors in liver responsible for its protective effect. Immature female rats of the Long-Evans strain were weaned at 21 to 23 days of age and fed *ad libitum* the following 3 diets: (1) diet A, (2) diet A plus liver concentrate powder 1-20<sup>7</sup> added at a level of 4% of the ration, and (3) diet A plus extracted liver residue<sup>7</sup> added at a level of 10%. The liver fractions were added in place of an equal amount of sucrose. All diets were supplemented with 500 mg of atabrine per kilogram of diet. Feeding was continued for 8 weeks (10 animals per group).

The findings indicate that the protective factor or factors is either water-insoluble or chemically bound so that it may not be readily removed by simple water extraction. No significant difference in growth or gross appearance was observed between atabrine-fed rats on diet A and those receiving liver concentrate powder 1-20 (containing the water-extractable material of raw liver). On the other hand, extracted liver residue (consisting of the coagulated, water-insoluble material remaining after the removal of the extractable water-soluble substances) was virtually as effective as whole liver powder in counteracting atabrine toxicity in the rat. Animals fed the above rations made the following gains in weight during the 8-week feeding period: diet A,  $41.6 \pm 10.4$  gm; diet A plus liver concentrate powder,  $52.8 \pm 9.7$  gm; diet A plus extracted liver residue,  $92.7 \pm 9.3$  gm.

#### DISCUSSION

Available data indicate that factors are present in liver and yeast that will counteract, at least in part, the effects of drug

<sup>6</sup> Heart rates averaged 504 to 537 per minute for all groups with an individual range of 465 to 570 per minute. The QRS interval averaged 0.02 seconds for all groups.

<sup>7</sup> We are indebted to Dr. David Klein of the Wilson Laboratories, Chicago, Ill., for the liver concentrate powder 1-20 and the extracted liver residue employed in the present experiment.

toxicity in the rat. As early as 1922 Funk expressed the view that the composition of the diet and perhaps its vitamin content may have a profound influence on the toxicity of drugs. This suggestion has been amply confirmed, not only in regard to the known nutrients (Ershoff, '48) but to additional factors present in liver or yeast. The beneficial effects of the latter in animals inhaling carbon tetrachloride or fed toxic doses of strychnine, promin, dinitrophenol, sulfanilamide and other drugs have long been recognized (De Santibañez, '43; Battelli, '40; Higgins, '44; Chamelin and Funk, '43). Similar results have been observed following toxic doses of diethylstilbestrol (Funk and Funk, '39; Chamelin and Funk, '43), estrogen (Engel and Rosenberg, '45; Ershoff and Deuel, '46) and desiccated thyroid (Ershoff and Hershberg, '45; Ershoff, '47a, b). The present experiment indicates that atabrine toxicity may also be counteracted, at least in part, by the administration of whole liver or yeast.

Our findings indicate that the effects of atabrine administration in the immature female rat are dependent on the diet employed. With rats fed a synthetic ration (diet A), administration of atabrine at a level of 500 mg per kilogram of diet resulted in marked retardation of growth, alopecia, inhibition of ovarian development, enlarged submaxillary glands, granulocytosis and myocardial damage as indicated by electrocardiographic tracings. The above effects were largely counteracted by the administration of desiccated whole liver at a level of 10% of the diet in place of an equal amount of sucrose. On this latter ration (diet D<sub>2</sub>) growth was significantly greater than on diet A<sub>2</sub>; alopecia did not occur; ovaries and submaxillary glands appeared normal both in weight and microscopic appearance; granulocyte counts were normal; and electrocardiograms were free of the abnormalities observed on diet A<sub>2</sub> although some prolongation of the PR interval was observed. Similar results were obtained with diet C<sub>2</sub> (in which yeast was fed in place of the whole liver), although growth was less than on diet D<sub>2</sub> and ovaries resembled in weight and microscopic appearance those of an



immature rat. Electrocardiograms in this series, however, remained free of the abnormalities observed in rats on other atabrine-containing rations and were indistinguishable from those of normal controls. Findings for rats on diet B<sub>2</sub> were intermediate between those for animals on diet A<sub>2</sub> and rations containing whole liver and yeast. Three of the 10 rats on this diet failed to grow; they developed alopecia and at autopsy revealed markedly enlarged submaxillary glands. The remainder of the series, however, were indistinguishable grossly or in microscopic appearance from animals fed yeast (diet C<sub>2</sub>). Electrocardiographic tracings revealed a marked elevation of the ST segment similar to that observed in 5 out of the 9 rats on diet A<sub>2</sub>. On atabrine-free diets no abnormalities were observed on any of the rations employed.

Since the toxic effects of atabrine were less pronounced in rats on diet B<sub>2</sub> than in those on diet A<sub>2</sub>, and since these 2 diets differed only in their content of known B vitamins, it is apparent that the beneficial effects of diet B<sub>2</sub> were due to its increased content of B vitamins. The latter ration contained twice the thiamine, riboflavin, pyridoxine, pantothenate and niacin content of diet A as well as significant amounts of biotin, folic acid, inositol and p-aminobenzoic acid. It would appear that one (or more) of the latter factors was responsible for the observed effects. However, the toxic effects of atabrine were still more pronounced on diet B<sub>2</sub> than on yeast or whole liver-containing rations (diet C<sub>2</sub> or D<sub>2</sub>). These findings would indicate that in addition to the known B vitamins there were still other factors in the whole liver and yeast that counteracted, at least in part, the effects of atabrine toxicity in the rat. Preliminary results indicate that the protective factor(s) is present in the water-insoluble fraction of liver remaining after the removal of the extractable water-soluble material.

#### SUMMARY

The administration of toxic doses of atabrine to immature female rats maintained on a synthetic ration resulted in marked retardation of growth, alopecia, inhibition of ovarian

development, enlarged submaxillary glands, granulocytosis and myocardial damage as indicated by electrocardiographic tracings. These effects were largely counteracted by the addition of desiccated whole liver or yeast to the basal ration and, to a lesser extent, by the administration of additional B vitamins. Whole liver was more effective than yeast or the additional B vitamins in promoting growth and ovarian development in the immature atabrine-fed rat. On atabrine-free rations no abnormalities were observed on any of the diets employed. The protective factor(s) was present in the water-insoluble fraction of whole liver. The suggestion is made that in addition to the known B vitamins, still other factors are present in whole liver and yeast that are required in increased amounts by the atabrine-fed rat.

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## SELF SELECTION OF DIET

### VII. THE EFFECT OF AGE AND PREGNANCY ON SELECTION <sup>1</sup>

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#### TWO FIGURES

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In a previous experiment, rats 6 to 9 weeks old were allowed their choice of casein, hydrogenated vegetable oil, sucrose, and a salt mixture (Scott, '46). About 40% of the animals refused to eat casein and a majority disliked sucrose as a choice. The present experiments describe the choices of younger (3 to 6 weeks old) and older (12 to 15 weeks old) animals and of animals kept on selection experiments for a long period of time.

Richter and Barelare ('38) reported that increased relative proportions of sodium chloride, olive oil, and casein were selected by animals during pregnancy, and believed these to be associated with increased need for these substances. In the experiments reported here, only an increased relative intake of salt mixture was found during pregnancy.

#### EXPERIMENTAL

In the first experiment, 15 male and 16 female albino rats were weaned at exactly 21 days of age, and allowed their choice of casein <sup>2</sup> hydrogenated vegetable oil, <sup>3</sup> sucrose, and a

<sup>1</sup> Contribution no. 656 of the Department of Chemistry, University of Pittsburgh. Aided by grants of the Nutrition Foundation, Inc., and the Buhl Foundation.

<sup>2</sup> Labco "vitamin-free."

<sup>3</sup> Primex.

salt mixture (Jones and Foster, '42), each in a separate cup. Vitamins were fed separately as pills.<sup>4</sup> The amount eaten from each cup was determined daily and the cups then interchanged in a predetermined random manner. This procedure was continued for 54 weeks or until the animal died.

In a companion experiment, 10 male and 10 female litter-mates of the rats used above were weaned at 21 days of age, and given a standard diet<sup>5</sup> plus vitamin pills until 12 weeks old, at which time the average weight of the males was 245 gm and that of the females, 181 gm. They were then allowed the same choices as in the first experiment and the same procedure followed for a 3-week period.

In the pregnancy study, rats from the first experiment were mated when 3 to 10 months old. Twelve successful matings were obtained. Choice of food of the females was recorded in the same manner as above for the 3 weeks of pregnancy and the preceding 3 weeks.

## RESULTS

Weanling rats showed a marked lack of ability to select an adequate diet. During the first 3 weeks only 9 rats out of 31 ate enough casein to gain weight, and one of these stopped eating it in the second 3-week period. One rat which ate no casein in the first 3 weeks began eating it in the fourth week. As a result, 22 of the 31 rats in the experiment died of lack of dietary protein at an average period of 37 days after weaning. The choices of the weanling animals during the first 3 weeks are shown in table 1, where they are compared with results on older animals. For tabulation purposes the animals were divided into those that failed and those that succeeded in gaining weight (groups A and B, respectively).

<sup>4</sup> One pill was given each rat daily. It contained approximately: 60  $\mu$ g thiamine hydrochloride; 120  $\mu$ g riboflavin; 90  $\mu$ g pyridoxine hydrochloride; 150  $\mu$ g calcium pantothenate; 10 mg choline chloride; 1 mg  $\alpha$ -tocopherol; and 55 I.U. vitamin A and 11 I.U. vitamin D as 0.001 ml Natola; all in a dextrin-powdered sugar base.

<sup>5</sup> The standard diet consisted of 24% casein, 10% Primex, 62% sucrose, and 4% salt mixture (Jones and Foster, '42).

Three males and 6 females from this group lived for a long period of time. One female died in the thirty-eighth week on selection, after failing to eat protein while pregnant. One male died in the thirty-fifth week, and 1 female died in the fifty-fourth week, both of unknown causes. The average growth and food selections of these 9 animals are shown in figure 1.

TABLE 1  
*Weight change and food selection of rats at various ages.<sup>1</sup>*

	NUMBER OF ANIMALS	CHANGE IN BODY WT.	TOTAL CALORIES EATEN	PER CENT OF TOTAL CALORIES			SALT EATEN
				Fat	Sucrose	Protein	
		<i>gm</i>					<i>gm</i>
GROUP A							
3-6 weeks old	22	— 9.3	256	84.7	13.5	1.9	1.2
6-9 weeks old <sup>2</sup>	34	— 21.0	403	76.6	22.3	1.3	0.9
12-15 weeks old	7	— 32.3	1158	73.1	21.6	5.4	1.9
GROUP B							
3-6 weeks old	9	47.1	513	61.2	13.4	25.5	5.1
6-9 weeks old <sup>2</sup>	53	58.9	877	46.7	25.9	27.4	5.4
12-15 weeks old	13	13.9	1264	41.7	32.6	25.8	5.9

<sup>1</sup> Data are 3-week averages.

<sup>2</sup> Results from another experiment (Scott, '46).

Thirteen out of 20 animals put on selection experiments at the age of 12 weeks ate protein and gained weight, while 7 did not. The selections of these animals are also shown in table 1.

With the exception of 1 male, successful matings were obtained with the 9 animals from the first experiment that were used in the pregnancy study. The average size of the 12 litters in the experiment was 8.1, and the young appeared to be of normal size and development. The young of only 1 female showed milk in their stomachs, and in every case the offspring were eaten within a few days. The selections during pregnancy and for the 3 preceding weeks are shown in figure 2. During the last 2 or 3 days before parturition, the mothers ate very little food — about 20 calories per day — and thus the average caloric intake for the final week of pregnancy was lower than that of the preceding week. The high rate of weight gain during the final week appeared then to be due in part to a change in the animals' water balance.

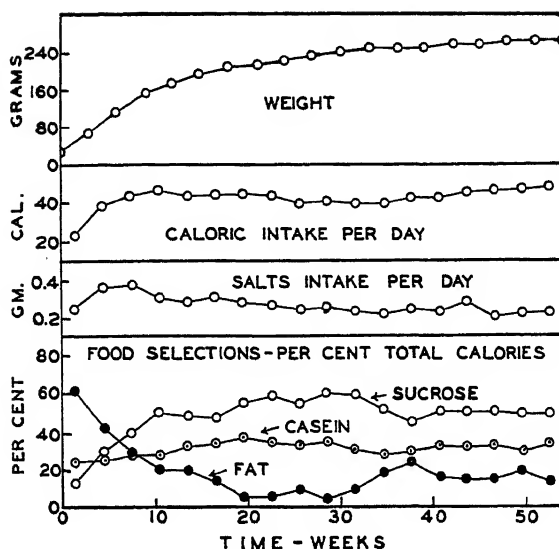


Fig. 1 Average weight and food selections of 9 rats over a long period. Each point represents an average for a 3-week period.

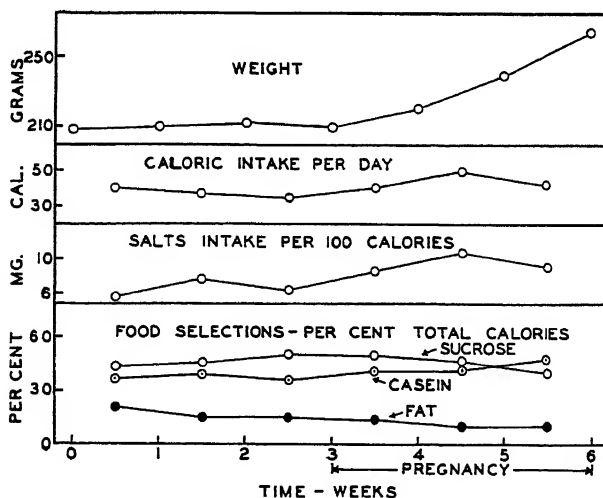


Fig. 2 Average weight and food selections before and during pregnancy. Each point represents a weekly average.

## DISCUSSION

The most obvious change associated with age in the choices of young rats was in the appetite for casein. The probability that as few as 9 out of a random sample of 31 animals would select casein was less than 1 in 1000, if the calculated probability of a rat selecting protein was 0.61. (The probability of an animal eating casein as determined with 6-week old animals was 0.61 [Scott, '46]). On the other hand, almost the same proportion of 12-week old animals (13 out of 20) selected casein, as was found with 6-week old animals (53 out of 87).

Another change in appetite with age was in the relative preference of young rats for fat and their relative avoidance of sucrose. Older animals had a greater preference for sugar and less for fat. In the long-term experiment, the only marked change in appetite was likewise a substitution of sugar for fat with advancing age. Neither of these 2 changes with age—protein appetite, and sugar versus fat appetite—appeared to have an obvious explanation.

The growth rate of the animals in the long-term experiment is of some interest, since, even disregarding the many animals which never grew, the rate is inferior to that of animals on a standard diet. At 12 weeks of age, 3 male rats on self-selected diets averaged 138 gm in weight, while 10 males on standard diet (12 weeks' experiment) averaged 245 gm. Similarly, 6 females on self selection averaged 167 gm, while 10 females on standard diet averaged 181 gm in weight. This is not in agreement with an earlier report (Richter, Holt and Barelare, '38).

During pregnancy, the food intake of the females increased, but the proportions of fat, sugar and casein eaten were almost constant. The intake of salts did increase relative to the caloric intake, however.

## SUMMARY

Only 9 out of 31 animals 21 days old selected casein and exhibited some growth when offered a choice of that protein,



sucrose, hydrogenated vegetable oil, and salts. The other 22 died at an average age of 58 days. About 60% of either 6-week old or 12-week old animals selected casein and gained weight. Younger animals showed more preference for fat and less preference for sucrose than older rats. During pregnancy, the intake of salts increased.

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## ERRATUM

WALTER C. RUSSELL, M. WRIGHT TAYLOR AND JAMES V. DERBY, JR. The folic acid requirement of turkey poults on a purified diet.

Journal of Nutrition vol. 34, no. 6, December, 1947. Page 632: second line from top of page to be changed to —

100 gm of purified diet (1.5 mg per kilo) for optimum growth



# THE EFFECT ON RAT GROWTH OF ALTERNATED PROTEIN INTAKES

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## ONE FIGURE

(Received for publication October 30, 1947)

Since the ultimate aim of much animal experimentation is the procurement of data applicable to man, experimental designs formally analogous to those common to human habit are of continuing interest. An initial experiment motivated by this concept has already been reported (Harte, '47).

Most small animal nutrition experiments are carried out with diets of constant composition. Human dietary practice, on the other hand, except, perhaps, in earliest infancy includes varied diet composition. To mirror human dietary vagaries experimentally would be an endless task; to simulate approximations within severely limited spheres is readily attainable.

Day-to-day variations in human food habits are innumerable. Not only may the several components ingested vary in quantity but also in quality. Further, numerous dietary components probably interact; many, no doubt, in ways not yet elucidated. These interactions are, in all likelihood, conditioned variously by the make-up of the diet as a whole.

This report presents data obtained from rat growth studies in which animals were fed, on alternate days, isocaloric rations containing different quantities of the same protein; in 1 experiment, protein quality as well as quantity was alternated.

## EXPERIMENTAL

Groups of randomly chosen weanling male rats maintained under conditions similar to those previously described (Harte et al., '47) were fed, *ad libitum*, rations made up to contain differing quantities of protein. Ration composition with respect to fats, salts, and vitamins was the same as that reported before; approximate caloric equivalence was attained by appropriate adjustments in content of cornstarch.

In 4 series of experiments, weighings were made every sixth day for 30 days; in the fifth series, weekly weighings were made for 4 weeks. The animals were not pre-conditioned by acclimatization to any synthetic ration since this practice has previously been shown to offer, at best, questionable advantage. (Harte et al., '47).

Food spillage was accounted for by scrupulous daily collection. The amount so collected was deducted from the apparent consumption of each ration and the protein intakes were calculated from the net food intakes.

## RESULTS

Examination of the data summarized in the upper part of table 1 shows that growth responses were virtually identical in the various comparisons involving different levels of the same protein. In the table, the first line of each pair of entries is to be taken as the control for the second. The 2 levels indicated in the second line of each pair represent the protein levels in the rations which were alternated; the control level, in every case, is the mean of the alternated levels. The growth curves in these instances coincided well throughout the experimental period and are not, therefore, reproduced.

On the other hand, the data in the lower part of table 1, where the animals in the alternated feeding group received not only different quantities of protein in the ration but protein differing in quality as well on alternate days, showed markedly different responses. The growth curves obtained under these circumstances are shown in figure 1.

TABLE 1  
*Growth responses of rats offered alternated diets.*

RATION FED	NO. OF ANIMALS	INITIAL WEIGHT	WEIGHT GAIN	FOOD INTAKE	PROTEIN INTAKE	PROTEIN EFFICIENCY <sup>1</sup>
		gm	gm	gm	gm	
Rations containing constant quality protein at different levels						
7% (control)	7	53.6 ± 2.0 <sup>2</sup>	32.6 ± 3.3	237 ± 12	15.9 ± 0.8	2.02 ± 0.15
5% and 9%	9	54.9 ± 1.9	32.8 ± 5.1	239 ± 11	16.2 ± 0.8	1.96 ± 0.21
10% <sup>4</sup> (control)	8	64.5 ± 2.4	61.6 ± 5.9	272 ± 19	27.2 ± 1.9	2.24 ± 0.12
5% and 15% <sup>4</sup>	8	57.4 ± 3.6	60.9 ± 2.1	268 ± 9	27.7 ± 0.8	2.20 ± 0.08
18% (control)	10	54.0 ± 2.1	108.4 ± 6.9	325 ± 12	58.5 ± 2.2	1.85 ± 0.08
12% and 24%	10	54.4 ± 1.6	115.1 ± 6.0	319 ± 13	57.2 ± 2.3	2.02 ± 0.07
20% (control)	10	53.8 ± 1.2	9.0 ± 2.2	175 ± 11	35.4 ± 2.2	0.239 ± 0.06
10% and 30%	10	51.4 ± 1.2	4.3 ± 1.9	162 ± 9	31.3 ± 1.8	0.121 ± 0.06
Rations containing protein mixtures of varying quality at different levels						
Wheat gluten, 10% + hydrolysate, <sup>5</sup> 5%	9	48.3 ± 2.2	65.4 ± 6.0	298 ± 19	44.7 ± 2.8	1.45 ± 0.07
Wheat gluten, 10% alternated with wheat gluten, 10% + hydrolysate, 10%	10	53.9 ± 1.9	46.7 ± 5.3	236 ± 13	35.4 ± 1.9	1.29 ± 0.12

<sup>1</sup> Protein efficiency is the ratio of weight gain to protein consumed.

<sup>2</sup> Indicated limits represent the standard errors of the means.

<sup>3</sup> Labco, from Borden Co.

<sup>4</sup> These tests were of 28 days' duration; the others continued for 30 days.

<sup>5</sup> The hydrolysate used was amino acids, a protein hydrolysate product manufactured by The Arlington Chemical Company.

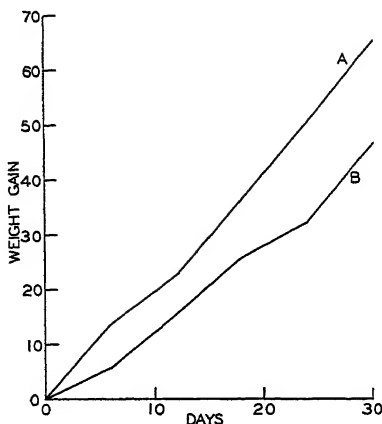


Fig. 1 Weight gain vs. time curves for animals on basic 10% wheat gluten diets supplemented (A) daily with 5% hydrolysate and (B) on alternate days with 10% hydrolysate.

#### DISCUSSION

The curve relating the variables protein efficiency and per cent casein in the ration is known to have a maximum at about 10–12% casein (Osborne, Mendel and Ferry, '19, and others). In view of this it is, perhaps, not surprising that alternations centered about a low level (7%), or a high level (18%) are without effect, as measured by weight gain or protein efficiency, when compared with appropriate controls. In the former case all of the levels are on the ascending portion of the curve; in the latter, on the descending side. On the other hand, it is hard to find an *a priori* explanation of the results of alternation about the 10% level, where the alternated levels are on either side of the maximum.

These data suggest that when a reasonably complete protein is fed, the rat has the capacity of integrating its intake over a 48-hour period. This finding is in marked contrast to that reported by Cannon et al. ('47) who found that a rat could not integrate its food supply when a missing amino acid was supplied 1 hour later, and to that of Elman ('39) who found that the dog could not utilize an intravenously administered

acid hydrolysate of casein when the missing tryptophane was introduced after 6 hours.

Another explanation which might be considered rests on the observation of Allison and associates ('46) that the nitrogen balance index of proteins is higher in depleted dogs than in normal animals. It is likewise true that depleted rats utilize protein more efficiently for growth (Travers and Harte, '47). The animals that were offered 5 and 15% casein might be regarded as being alternately depleted and repleted. The observed protein efficiency of this group is higher than might have been predicted on the basis of the protein efficiency vs. protein level curve for casein. If the protein intake on the 5% ration is inadequate to sustain the accelerated growth rate of the alternated 15% ration, then there would result an intermittent pseudo-depletion with an intermittent, concomitant better-than-normal utilization of the protein fed at the higher level. Frazier et al. ('47) have found that, in general, the effects of an incomplete amino acid ration are realized within 24 hours. So, also, a low level of protein, contrasted with an alternate high level, could be expected to manifest its effects in a similar period of time, but the overall effect, in this case, seems to be integrated over the unit interval of 48 hours.

That no significant effect was found in the wheat gluten experiment might have been anticipated from the report of Barnes et al. ('45). Our experiments are uncomplicated to the extent that the nature of the protein source was not varied in the several comparisons, merely the level. Where daily variation introduces the factor of differing quality as well, the possibility of other results could be entertained. This variation in quality as well as quantity constitutes a closer analogy to human habits.

The experiment summarized in the lower part of table 1 and figure 1 compares the response of the growing rat to a constantly supplemented poor protein diet (10% wheat gluten) with the response to intermittent supplementation of the same diet. To some extent this might simulate conditions apt



to prevail in the actual use of dietary protein supplements in medical practice. Here the efficiency of utilization for growth of dietary protein ( $N \times 6.25$ ) was not affected; the protein efficiencies do not differ significantly. However, the actual growth responses do show substantial divergence which is statistically significant ( $p = 0.03$ ).

For the protein hydrolysate product employed, an amino acid analysis (Block, '47) shows that its limiting amino acids are methionine and cystine (Block and Mitchell, '46-'47), while pair-fed weanling rats do as well on this material as on casein (Oser, '47). The deficiency of lysine in wheat gluten is well-known. In the alternation experiment then, it may be that the situation is more nearly akin to that observed by Cannon and coworkers ('47) and we are encountering a (partial) manifestation of the rat's inability to integrate a complete amino acid supply when all of the necessary amino acids are not offered simultaneously. This problem has recently been explored in the case of growing rats by Geiger ('47). He found that delayed supplementation of an incomplete protein by the missing amino acid (12-hour alternation) failed to promote growth.

It is noteworthy that the animals in the alternated group shown in the lower part of table 1 consumed hydrolysate almost exactly equivalent to one-third of their total protein intake, just as did the rats in the control group. The difference in total food intake in these groups is statistically significant ( $p = 0.02$ ) and this may well be the effect of the alternation and thus the cause of the difference in growth response.

#### SUMMARY

1. Weanling rats grow as well, and utilize dietary protein with the same efficiency for growth whether it is supplied at a constant level in the diet or whether the level is alternated daily about the same value as a mean, provided the protein quality is constant (casein or wheat gluten).

2. Daily alternation of both quality and quantity of dietary protein, using wheat gluten and a protein hydrolysate, results

in significantly poorer growth response but not significantly lowered protein efficiency.

3. The data suggest that prescribed dietary protein supplementation should be maintained constant to achieve maximum effectiveness from a given amount of supplement.

#### ACKNOWLEDGMENT

The assistance of Louis Quesal is greatly appreciated.

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# THE DIGESTIBILITY OF RAPE-SEED OIL IN THE RAT<sup>1</sup>

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There is general agreement that vegetable and animal fats which melt at 50°C. or lower are practically completely digested in man and in other animals. The chief exception to the complete digestibility of liquid fats in man is in the case of such irritants as castor or croton oil where the premature removal of the fat from the small intestine before the completion of lipolysis and absorption results in a lowered utilization.

However, there are species differences in fat availability since castor oil may be largely utilized in the rabbit, guinea pig and sheep where it exerts no cathartic action (McCay and Paul, '38; Paul and McCay, '42). McCay and Paul ('38) have also shown that guinea pigs are especially sensitive to high melting fats.

We became interested in a study of the digestibility of rape-seed oil because of the report by Boer et al. ('47) that rats grew less favorably on a diet containing rape-seed oil than on a diet containing butter. This finding has been corroborated in our laboratory (Deuel et al., '48). Rape-seed oil had previously been shown to be the slowest absorbed of a number of natural fats tested (Deuel et al., '40) so it might be suspected that the digestibility would also be the lowest

<sup>1</sup> This was aided by a research grant from Swift and Co.

of the common liquid fats. Although rape-seed oil is not used for edible purposes to any great extent in the United States, the cold pressed oil is widely used in Europe and especially in India for food purposes. It is used in England by bakers for greasing the ends of loaves of bread to prevent their sticking together (Lewkowitsch, '21).

It has been known for a long time that fat is excreted in the feces not only as the triglyceride and fatty acid which are ether-soluble but also in the form of soaps which are insoluble in ether. However, little attention has been paid to this latter fraction and the extensive series of tests of fat digestibility in man reported by Langworthy ('23) were based only on the fecal fat fraction which was directly soluble in ether. In recent tests on rats (Augur et al., '47; Crockett and Deuel, '47), it was shown that considerable amounts of ingested fats may be excreted as soaps. In the case of a number of low-melting fats such as margarine, hydrogenated cottonseed oil<sup>2</sup>, prime steam lard and bland lard, the soap fraction was quite low and its inclusion in the excreted fat did not appreciably alter the calculated values of the coefficient of digestibility since the correction for metabolic fat is correspondingly increased. On the other hand, in the case of hydrogenated lards melting at 61°C. as well as with several samples of hydrogenated cottonseed oil, the soap content greatly exceeded the neutral fat-fatty acid value in spite of the fact that the latter fraction was also usually considerably increased. However, in the tests on the hydrogenated lard melting at 55°C., the soap fraction was largely increased without any marked rise in the excretion of neutral fat and fatty acid. Cheng and Morehouse ('48) have shown that the proportion of fat excreted as soap is much higher when a calcium-magnesium rich salt mixture (Osborne-Mendel, '17) is included in the diet than on a control diet containing no added salts. It is thus apparent that an entirely false impression of the digestibility of fat may be gained if account is not taken of the soap fraction.

<sup>2</sup> Crisco.

## EXPERIMENTAL AND RESULTS

The experimental procedure was similar to that used by Augur et al. ('47). The diets containing the rape-seed oil were fed over 10-day periods. This included a preliminary 2-day orientation period followed by an 8-day interval during which the food consumption was measured and the feces were collected for fat analysis. The diet had the following percentage composition: commercial casein, 18; glucose, 56; rape-seed oil, 15; Osborne-Mendel ('17) salt mixture, 7; yeast,<sup>3</sup> 1; liver extract,<sup>4</sup> 3. Food was given ad libitum during the tests. The animals used were adult female rats from our stock colony. Total glyceride, fatty acids and soaps in the stools were determined by diethyl ether extraction of the dried stool. Soaps in the feces were determined by first converting them to fatty acids by acidification of the dried ether-extracted residue of the stools with a minimum quantity of 50% sulfuric acid; the acid paste was then re-extracted with ether.

The calculations for digestibility were made in the usual way after correction for the metabolic fat. This correction was determined in experiments carried out on a diet similar to the above except that the fat was replaced by glucose. The correction for metabolic fats used in these tests (65 mg per gram dried stool) is slightly higher than the value found by Augur et al. ('47) which was 50.5 mg.

The crude rape-seed oil used in the tests was a commercial product.<sup>5</sup> It was a dark thick viscous oil having a free fatty acid content of 0.6%. The refined rape-seed oil was prepared from the crude sample by bleaching and deodorizing<sup>6</sup>; it had a light yellow color, a satisfactory odor and a free fatty acid content of 0.01%. The following constants were found for the crude and the refined rape-seed oils, respectively: saponification number, 166.8 and 167.7; iodine number, 101.5 and 96.3.

<sup>3</sup> Anheuser-Busch, strain G.

<sup>4</sup> Wilson, 1: 20.

<sup>5</sup> From the Pacific Vegetable Oil Co., San Francisco.

<sup>6</sup> We wish to thank Mr. R. H. Neal of The Best Foods, Inc., for refining the rape-seed oil.

Two series of tests were carried out on rape-seed oil. Since the first tests on the crude oil sample gave the unexpectedly low coefficient of digestibility, the experiments were repeated with the highly-refined product. Ten rats were used in each series of tests. The results are summarized in table 1. The values on a margarine fat from earlier tests are included for comparison.

TABLE 1

*Summary table of digestibility of crude and refined rape-seed oil compared with margarine fat when fed to female rats in a diet at 15% level.*

DATA SUMMARIZED	CONTROL TESTS	CRUDE RAPE-SEED OIL TESTS	REFINED RAPE-SEED OIL TESTS	MARGARINE FAT TESTS <sup>1</sup>
Number of rats	10	10	10	10
Average weight of rats, gm	170	175	160	214
Average gain, gm	— 4	4	— 1	— 1
Fat in diet, %	0	15	15	15
Average food eaten, gm	69	80	52	72
Average fat ingested, gm	0	12.1	7.8	10.7
Average weight of dried stools, gm	3.83	6.47	3.43	4.40
Average fat excreted				
As neutral fat and fatty acids, gm	0.12	1.07	0.65	0.24
As soaps, gm	0.12	2.12	0.99	0.30
Total, gm	0.25	3.19	1.64	0.54
Total, corrected, gm <sup>2</sup>		2.77	1.42	0.31
Coefficient of digestibility		77	82	97
Standard error of mean <sup>3</sup>		1.6	1.5	0.4

<sup>1</sup>Data from paper of Angur et al. ('47).

<sup>2</sup>Corrected for metabolic fat by multiplying stool weight by 65 mg.

<sup>3</sup>Calculated by the formula  $\sqrt{\Sigma d^2/n-1} / \sqrt{n}$  where "d" is the deviation from the mean and "n" is the number of observations.

## DISCUSSION

Rape-seed oil is the first fat, liquid at ordinary temperatures, for which such a low coefficient of digestibility as 77 has been found. This value is only slightly improved with a highly refined rape-seed oil and therefore must be attributed to the fat itself rather than to impurities present in the crude product. These results are in line with earlier tests (Deuel et al., '40) where it was shown that the rate of absorption of

rape-seed oil from the intestine was the slowest of any of a number of natural fats.

The poor digestibility of rape-seed oil must be related to its composition. This fat consists of 40 to 50% of trierucin and not more than 5% of the glycerides of saturated acids, with the balance being made up of triolein and trilinolein (Hilditch, '40). The feature which distinguishes it from the other vegetable oils having high digestibilities is the high content of trierucin. It is apparent that this triglyceride is largely hydrolyzed in the gastrointestinal tract as the principal fat excretion is in the soap fraction. Therefore, it would seem logical to assume that the difficulty in digestibility was associated with the poor absorption of erucic acid.

The reason for the discrepancy between the tests on rats and the earlier results on man (Holmes, '18) is not immediately apparent. Holmes reported an average coefficient of digestibility of 98.8 on 4 subjects who consumed an average of 82 gm of rape-seed oil daily. It is possible that this variation may be ascribed to a species difference. Another possible explanation may be that had the values obtained by Holmes ('18) been corrected for the fat lost as soap, a much lower coefficient of digestibility would have been recorded. The answer to this question can only be obtained by new experiments on human subjects where a determination of the excretion of fat in the form of soaps is made. Because of the widespread use of rape-seed oil as a food, it would seem to be of considerable importance that such tests be made on man.

#### SUMMARY

Rape-seed oil has been found to have the lowest coefficient of digestibility of any fat, liquid at ordinary temperature, which has been investigated on rats. Crude rape-seed oil was digested to the extent of only 77% while the refined oil gave an average value of 82%. This apparently cannot be traced to a failure of lipolysis but is believed to be related to the poor absorbability of the erucic acid fraction.



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# STUDIES ON THE COMPARATIVE NUTRITIVE VALUE OF FATS

## X. ON THE REPUTED GROWTH-PROMOTING ACTIVITY OF VACCENIC ACID <sup>1</sup>

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In a recent paper (Boer, Jansen and Kentie, '47), data were presented in support of the view that summer butter possesses a growth-promoting activity other than vitamin A, which is not shared by winter butter or by the vegetable oils. The factor responsible for this effect was found in the unsaturated fatty acid fraction and was identified as vaccenic acid. In another report (Boer, Jansen, Kentie and Knol, '47) vaccenic acid, prepared from hydrogenated China wood oil, was tested under similar conditions and was shown to produce the growth-promoting effect. It was therefore concluded that the factor in summer butter responsible for this activity is vaccenic acid. The earlier experiments of Boer and Jansen ('41) which indicated a superiority of butter over olive oil in producing growth in rats were not confirmed by Euler and his coworkers ('42, '43), but Boer, Jansen and Kentie ('47) at-

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The authors wish to thank the Winthrop Chemical Co. for the crystalline vitamin D<sub>2</sub> and Merck and Co. for the  $\alpha$ -tocopherol.

tribute this to the absence of vaccenic acid in the winter butter of Sweden.

In earlier studies from this laboratory (Deuel et al., '44a), no differences could be demonstrated in the growth rate of young rats when fed on diets complete in the known nutritional requirements irrespective of whether corn, cottonseed, olive, peanut or soybean oil, a margarine, or a summer butter were incorporated in the diet. Similar results were obtained by Zialeiti and Mitchell ('44) and by Deuel and Movitt ('45) when the test animals were rats which had been prematurely weaned. Such animals had been previously reported as being especially sensitive to a lack of an essential factor present in butter although it was stated that this sensitivity was lost after 6 weeks if the animals were maintained on a stock diet (Schantz, Elvehjem and Hart, '40; Schantz, Boutwell, Elvehjem and Hart, '40). In later tests from the present laboratory (Deuel et al., '46) vegetable oils, a margarine or a butter were found to be equally satisfactory in the diet in protecting against undernutrition or in satisfying the dietary needs for augmented growth which could be produced by the injection of the growth hormone.

Euler et al. ('46) have reported as satisfactory growth on a margarine fat mixture as on butter in rats over a 700-day period. Superior fertility and lactation along with a somewhat lowered mortality resulted from the margarine diet. These authors reported that the total weight of all offspring at 30 days in series A from 25 females on the butter diet was 8508 gm, compared with 15,955 gm for a like number of females receiving the margarine diet, and 15,047 gm for the animals receiving the stock diet. In series B, similar results were obtained although the performance of both the butter and margarine groups was improved by the addition of vitamin E to the diet. The total weight of all offspring at 28 days of age was found to be the following for series B: butter diet, 5422 gm; margarine diet, 8186 gm; butter diet + vitamin E, 7613 gm; and margarine diet + vitamin E, 9494 gm. Deuel et al. ('44b) reported no differences in reproduction or lactation

where both of the parents had been raised on the several vegetable fats or a margarine as compared with the diet containing butter. Euler points out that our failure to demonstrate a superiority for the vegetable fats was because our tests were carried out over a shorter period and with standardized litters.

The vegetable oils employed in our earlier tests were those produced and used for dietary purposes in the largest amounts in the United States. These included corn, cottonseed, olive, peanut and soybean oils. On the other hand, Boer et al. have used rape-seed oil and olive oil for their comparison with butterfat, although peanut oil was used in some of the preliminary tests. It was noted that "growth on rape-seed oil and on olive oil not only diminished at an earlier time than on peanut oil, but also that the difference in growth between the animals receiving butter and the control rats was greater than when peanut oil was used."

The present tests were undertaken to determine (1) whether any augmentation in growth could be demonstrated when vaccenic acid was used as a supplement to a typical vegetable oil such as cottonseed, and (2) whether the inferior growth obtained with the rape-seed oil diets might be ascribed to a lowered food consumption by rats on such diets. Finally, the tests were designed to determine whether any growth-promoting activity could be demonstrated with vaccenic acid of high purity.

#### EXPERIMENTAL

Twenty litters of our stock rats (Wistar strain) were used with litter mates equally distributed between the 6 dietary groups. Each group consisted of 20 rats (10 males and 10 females except for group IV in which there were 11 males and 9 females). The rats were placed on the diets at 29 days of age. Food was given ad libitum. The reserve diets were kept in the refrigerator except at time of feeding. In addition, all rats received as a supplement a daily oral dose of 0.1 ml of the same fat as that present in the diet which they were receiving. This included vaccenic acid (groups II and VI) or China wood oil (group III) in solution. In order to avoid the

criticism that variations in growth could be attributed to the administration of the fat supplements, similar amounts of the particular fat under investigation were administered irrespective of whether or not it contained vaccenic acid or hydrogenated China wood oil.

The diets used were essentially the same as those of Boer, Jansen and Kentie ('47). The composition is given in table 1, while the constants for the fats used are summarized in table 2.

TABLE 1  
*Composition of the diets used.*

DIET CONSTITUENT	GROUP					
	I	II	III	IV	V	VI
	%	%	%	%	%	%
Whole ground wheat	72	72	72	72	72	72
Commercial casein	5	5	5	5	5	5
Yeast <sup>1</sup>	10	10	10	10	10	10
Salt mixture (Osborne-Mendel, '17)	3	3	3	3	3	3
Rape-seed oil <sup>2</sup>	10	10	..	..	..	..
Rape-seed oil containing 3% hydrogenated China wood oil <sup>2</sup>	..	..	10	..	..	..
Butterfat <sup>2,3</sup>	..	..	..	10	..	..
Cottonseed oil <sup>2,4</sup>	..	..	..	..	10	10

Following supplements also given daily (6 times weekly) by mouth: I, 0.1 ml rape-seed oil; II, 0.1 ml rape-seed oil containing 10 mg vaccenic acid; III, 0.1 ml rape-seed oil containing 3% China wood oil; IV, 0.1 ml butterfat; V, 0.1 ml cottonseed oil; VI, 0.1 ml cottonseed oil containing 10 mg vaccenic acid.

<sup>1</sup> Anheuser-Busch, strain G.

<sup>2</sup> The following supplements were added to all oils per 100 gm oil: carotene (General Biochemicals, Inc.) 2.4 mg; fish liver oil containing 350,000 I.U. per gram, 19.2 mg; crystalline vitamin D<sub>2</sub>, 0.05 mg;  $\alpha$ -tocopherol, 36.0 mg; commercial butter flavor, 1.2 mg.

<sup>3</sup> Challenge Brand obtained locally.

<sup>4</sup> Wesson oil.

Following the unexpected finding that pure vaccenic acid is something less than a nutritional supplement in the diet of the rat, it occurred to us that this singular effect might have been due to the presence of detrimental quantities of lead in our product, the last traces of which are removed only by very

TABLE 2  
*Constants of the fats and vaccenic acid used in the tests.*

FAT OR FATTY ACID	SAPONIFI- CATION NUMBER <sup>1</sup>	IODINE NUMBER (W136) <sup>1</sup>	NEUTRALI- ZATION EQUIV.	MELTING POINT	REFRACTIVE IN- DEX, ABBE 70° C.	MICROANALYSIS	
						Carbon	Hydrogen
Vaccenic acid				° C.		%	%
Theoretical or literature	.....	89.9	282.3	Various	1.4432 <sup>2</sup>	76.51	12.10
First lot	.	88.4	283.2	38-39			
Second lot	.	87.4	282.8	inc.	1.4415	77.61 <sup>4</sup>	12.63 <sup>4</sup>
Crude rape-seed oil	166.8	101.5		38.4 <sup>3</sup>	1.4434	76.96 <sup>4</sup>	12.42 <sup>4</sup>
Refined rape-seed oil	167.7	96.3		38.8 <sup>3</sup>		76.34 <sup>5</sup>	12.04 <sup>5</sup>
Butterfat	228.6	33.2					
Cottonseed oil	193.7	111.1					
Hydrogenated China wood oil	184.7	77.5					

<sup>1</sup> We wish to thank Amber L. S. Cheng for determining the constants on the several fats.

<sup>2</sup> Boescken et al. ('30).

<sup>3</sup> Mixed melting points were unchanged.

<sup>4</sup> Analyses at California Institute of Technology by the courtesy of Dr. A. J. Haugen-Smit.

<sup>5</sup> Analyses at Columbia University through the courtesy of Professor H. T. Beans.

thorough acid washing. However, quantitative spectrographic analyses indicated that this was not the case.

The crude rape-seed oil was a commercial product<sup>2</sup> that had been refined, bleached, and deodorized before being shipped to us for the tests. It had a light color, 0.01% of free fatty acids (compared with 0.6% in the crude oil) and only a slight taste.

Although the butter used in the tests was purchased on the local market, it was unquestionably a summer butter. Cattle in this area are on green feed most of the year. An analysis of the present butter sample for carotene gave a total value of 7.2  $\mu$ g of yellow pigment per gram of butter. No artificial coloring was present. From the saponified butter sample,  $\beta$ -carotene was separated by chromatography; on extraction from the column, this amounted to 5.2  $\mu$ g per gram of butter. It gave an absorption curve typical of  $\beta$ -carotene as determined spectrophotometrically. These values are in the normal range of carotene in commercial butters (Fraps and Kemmerer, '43) and exceed those of Holstein cows on summer feed (Deuel et al., '42). One must conclude that the butter used was a "summer butter."

## RESULTS

The results on the 6 groups of rats are summarized in table 3. The gain in weight of all groups receiving rape-seed oil (I-III) is in most cases significantly lower than the increase in weight of the animals receiving butterfat (IV) or cottonseed oil (V). There is no evidence that the supplementation with vaccenic acid improves the growth of the rats receiving rape-seed oil (II) or of those which were on a basal diet containing cottonseed oil (VI). In fact, there would seem to be a depressing effect on growth in the male rats in both of these groups. The results with hydrogenated China wood oil are also negative as far as growth-promoting activity is con-

<sup>2</sup> Pacific Vegetable Oil Co., San Francisco. The sample was refined, bleached, and deodorized in the Bayonne Laboratories of the Best Foods, Inc., after which it was shipped to us for the tests. We wish to express our appreciation to Mr. N. G. Barradas of the San Francisco branch of The Best Foods, Inc. for obtaining this sample of crude rape-seed oil for us.

cerned. A statistical analysis of the data shows that the ratio of mean difference to standard error of mean difference exceeds the significant value of 2.5 where groups I, II or III (males) are compared with groups IV or V (males) but in no other case.

Because of the considerable variation in starting weights in the several groups, a recalculation was made for 8 male and 8 female rats in each group so that the average initial weights would have a spread of less than 2 gm. Selections of the rats to be dropped from consideration were based solely on their starting weights. Where the original level of body weight was too low, the 2 rats with lowest weights were dropped [males I, II, III, VI; female IV (1 rat only)]. Where the beginning weights were too high, the rats with the highest starting weights were dropped from consideration (male V; females I, II, III, VI). The 2 lowest weight rats and one with average weight were dropped from the males in group IV, while the 2 rats with lowest and highest weights at the start were deleted from group V (females). The gain in weight in this revised group in no way alters the picture.

The food consumption of the rats was determined over the 6-week period. The average weight of the food ingested and the efficiency of utilization are reported in table 3. A higher efficiency of utilization of the diets by the male rats as compared with the females confirms our earlier observations (Deuel et al., '44a). When the caloric values of the diets are corrected for the utilizable fat, little difference in efficiency of utilization of the diets is to be observed, using the data for the "selected group."

#### DISCUSSION

The present experiments demonstrate that whether the diet contains a vegetable oil such as cottonseed oil, or butterfat, the growth of the rats is identical. In the present tests, the other components of the basal diet were whole ground wheat, commercial casein, yeast, salt mixture, and a mixture of fat-soluble vitamins. These results also confirm our earlier re-



port (Deuel et al., '44a) where the basal diet consisted of skimmed milk powder and the fat (vegetable oils, a vegetable oil margarine or a butter) fortified with the fat-soluble vitamins.

The present tests also confirm the results of Boer, Jansen and Kentie ('47) which show a superior growth for butterfat as compared with rape-seed oil. However, the results on rape-seed oil are not typical of vegetable oils in general because cottonseed oil (present report) and corn, cottonseed, olive, peanut and soybean oils (Deuel et al., '44a) all give a growth response identical with that for butter.

There are several possible explanations for the atypical results with rape-seed oil. In the first place, the crude product has an especially unappetizing taste which would be expected to cause a lowering of the food consumption with a resulting decreased growth rate. Although no data on food consumption are available from the Boer et al. experiments, the food consumption values for the rape-seed groups in the present tests are somewhat lower with the males but not with the females, even where a highly refined product was used.

It would also appear that the rape-seed oil diets have a lower efficiency of utilization. This is probably to be traced to the unsatisfactory digestibility of the rape-seed oil. Although it has generally been considered that all fats melting at 50°C. or lower are utilized to an extent of 95% or more, Deuel, Cheng and Morehouse ('48) have found that the coefficient of digestibility for crude rape-seed oil in the rat is 77.2 while that of the refined oil is only 82.5. When corrections are made in calculating the caloric values of the diets for the digestibility of the fats, the differences in the efficiency of utilization between the rape-seed oil diets, on the one hand, and the butterfat and cottonseed oil diets, on the other, largely disappear. It would thus seem evident that the lower growth rate on the rape-seed oil diets is to be ascribed mostly to the poor digestibility of the rape-seed oil as compared with the practically complete digestibility of butterfat and cottonseed oil.

TABLE 3

Summary table of mean starting weights, weight gains, and food consumption of rats receiving a diet containing rape-seed oil (I), rape-seed oil + vaccenic acid (II), rape-seed oil + hydrogenated China wood oil (III), butterfat (IV), cottonseed oil (V), or cottonseed oil + vaccenic acid (VI) over a 6-week period.

DIET NO.	STARTING WT. AT 29 DAYS OF AGE				GAIN DURING 6 WEEKS				FOOD INTAKE IN 6 WEEKS				DIET EFFICIENCY <sup>2</sup>		
	M	P	Aver- age	gm	M	P	Aver- age	gm	M	P	Aver- age	gm	M	P	Aver- age
I	64.6 ± 2.7	66.4 ± 2.6	65.5	152.0 ± 8.3	91.3 ± 3.6	121.6	484.3	452.0	7.69	5.33	6.59				
II	63.2 ± 3.2	66.4 ± 3.2	64.8	144.4 ± 7.0	91.4 ± 5.0	117.9	423.3	448.1	7.49	5.29	6.45				
III	66.8 ± 2.3	63.2 ± 2.6	65.0	158.0 ± 3.5	92.3 ± 3.5	195.2	527.9	474.3	7.34	5.38	6.47				
IV	65.5 ± 3.5	57.8 ± 2.4	61.7	183.9 ± 3.5	101.0 ± 3.5	142.5	540.2	474.3	8.34	6.06	7.36				
V	71.7 ± 3.2	60.1 ± 3.2	65.9	178.6 ± 5.0	103.7 ± 6.0	141.2	554.5	481.8	7.90	6.22	7.18				
VI	64.2 ± 1.8	68.3 ± 3.3	66.2	169.2 ± 5.4	101.5 ± 2.8	135.4	526.1	482.9	7.85	5.66	6.87				
Calculations based on 8 males and 8 females — "selected group"															
Ia	68.1 ± 2.7	63.5 ± 1.8	65.8	154.5 ± 6.0	92.4 ± 4.2	123.5	495.1	455.9	7.98	5.67	6.93				
IIa	65.6 ± 3.5	63.1 ± 2.5	64.4	149.7 ± 6.0	92.7 ± 5.8	121.2	490.6	455.9	7.79	5.63	6.80				
IIIa	69.3 ± 2.0	60.4 ± 2.0	64.9	158.1 ± 4.3	88.4 ± 4.0	123.2	529.5	465.3	7.64	5.63	6.78				
IVa	69.0 ± 3.3	59.4 ± 1.3	64.2	186.6 ± 4.4	99.0 ± 3.3	142.8	554.5	481.8	8.31	5.97	7.32				
Va	68.5 ± 2.6	60.1 ± 2.6	64.3	179.4 ± 5.7	105.8 ± 7.5	142.6	558.5	486.9	7.93	6.29	7.23				
VIa	66.3 ± 1.5	64.4 ± 2.3	65.4	162.5 ± 2.8	101.0 ± 3.6	131.7	516.6	475.4	7.77	5.74	6.84				

Each original group contained 20 rats (10 males, 10 females except group IV which had 11 males and 9 females).

<sup>1</sup>Including the standard error of the mean calculated by the formula  $\sqrt{2d^2/n-1}/\sqrt{n}$  where "d" is the deviation from the mean, and "n" is the number of observations. Because of wide variations in the gain of weight of males and females, the value would be meaningless for the combined groups.

<sup>2</sup>Ratio of gm gain x 100. Calculated on basis of calorie values of the diets being 4.08 Calories per gram.

<sup>3</sup>For the revised groups (Ia-VIa) the same ratio as above was used but based on following calorie values of diets:

Rape-seed oil diet — 3.91 Calories per gram (based on 82% digestibility of fat).

Butter and cottonseed oil diets — 4.05 Calories per gram (based on 97% digestibility of fats).

There is no evidence from the present tests of any improved growth when either purified vaccenic acid or hydrogenated China wood oil is added to the diet. In this observation, our results are at variance with those of Boer, Jansen and Kentie ('47) as well as Boer, Jansen, Kentie and Knol ('47). It is not known whether these investigators used a crude or a refined rape-seed oil. In the former case, a masking of the unappetizing flavor of the oil might have increased food consumption although there are no data in their reports on the amount of food consumed. No change in the food intake was noted in our tests where a refined rape-seed oil was used whether the diets were supplemented or not with vaccenic acid or hydrogenated China wood oil.

Vaccenic acid apparently cannot be identical with the factor which Schantz, Elvehjem and Hart ('40) have indicated as being present in butter. The latter factor was believed to be a long chain *saturated* acid while vaccenic acid belongs to the group of *unsaturated* acids. There was no indication that the content of the factor of Schantz et al. varied in summer and winter butter. Vaccenic acid is believed to be present only in summer butter. Finally, the need for the Schantz et al. factor was only apparent during the period immediately following weaning at 21 days of age; the depletion for the vaccenic acid tests was continued 3 or 4 weeks after weaning at 28 days, by which time Schantz and associates found that most of the differences between the various fats had disappeared.

The experiments of Jack et al. ('45) as well as those of Henry et al. ('45) indicate that there is no one fraction of butterfat the growth promoting action of which is superior to that of whole butter. However, Geyer et al. ('47a) have recently reported a superior growth-promoting action of the liquid fraction of butter remaining after the separation of the solid portion from an acetone solution at  $-4^{\circ}\text{C}$ . They obtained positive results only on a single sample of September, 1945 butter but they were unable to confirm it on June or December, 1945 samples as well as on butters obtained during

February, June, July or September, 1946. Food consumption was not determined for the single positive test.

Although there would seem to be some evidence of the presence of vaccenic acid in butter and animal fats (Bertram, '28; Grossfeld and Simmer, '30; Geyer et al., '47b), and also in margarine (Bertram, '28; Grossfeld and Simmer, '30) as well as its absence in vegetable oils (Geyer et al., '47b), the evidence is somewhat weak in view of the fact that the  $\Delta^{12, 13}$ -octadecenoic acid which melts at 39.5° and 39.7–40.1°C. (Richter, '42) has not been excluded. There is no indication from the present experiments that vaccenic acid itself possesses any specific function in growth.

#### SUMMARY

1. No differences in the rate of growth of male and female rats were noted over a 6-week period when the diet contained butterfat or cottonseed oil.

2. The growth rate of rats receiving rape-seed oil diets was somewhat less and the efficiency of utilization of these diets poorer than those obtained on the butter or cottonseed oil diets. It is believed that the less efficient utilization of the rape-seed oil diet is to be attributed to the poor digestibility of the fat which may be due to its characteristically high content of eracic acid ( $\Delta^{13:14}$  docosenoic acid).

3. No stimulating effect on growth was produced by the administration of vaccenic acid or hydrogenated China wood oil to the rats on the rape-seed oil diet. Moreover, no increased growth resulted in the cottonseed oil group when a supplement of vaccenic acid was fed.

4. It is concluded that vaccenic acid plays no specific role in relation to growth of the rat.

#### APPENDIX

*Preparation of vaccenic acid (Gooding and Brown<sup>3</sup>). Vaccenic acid was separated from partially hydrogenated China wood oil<sup>4</sup>*

<sup>3</sup> Research Laboratories, The Best Foods, Inc.

<sup>4</sup> One sample of the crude China wood oil was obtained from the Standard Varnish Co., Staten Island, New York, and the second sample from Werner G. Smith Co., Elizabeth, New Jersey.

by the method of Boeseken et al. ('30) with some modifications. The acidity of the crude oil was reduced by esterification with methanol prior to hydrogenation. In order to minimize polymerization and wandering of double bonds, the hydrogenation to iodine values ranging from 80 to 86 was accomplished in less than 10 minutes by use of 1% commercial nickel catalyst at 45 p.s.i. and at 100° to 200°C. The hydrogenated glycerides were converted to methyl esters by alkaline alcoholysis and the esters distilled to remove polymers. The fatty acids from the distilled esters were treated with lead acetate and the lead soaps were recrystallized once using the proportions of reagents recommended by Hilditch ('40).

In general terms, pure vaccenic acid was separated from the fatty acids of the lead soaps by first removing the major part of saturated acids by crystallization from methanol at about 8°C. The filtrate (I) was diluted with 5 volumes per cent of water and again held at 8°C., whereupon the remaining saturated acids and a large part of the vaccenic acid were obtained as a filter cake (II). The filtrate from II was then held at 0°C. to obtain the first crop of crude vaccenic acid (III). The filtrate from III was repeatedly reused as a solvent for filter cake II to obtain successive crops of crude vaccenic acid by repeating the sequence of crystallizations and filtrations already described. The volume of solvent varied from 3 to 15 times the weight of acids and all filtrations were done in the refrigerator after at least 16 hours, using precooled Buchner funnels fitted with a rubber dam. The yields of crude vaccenic acid were combined and finally purified by repeating the described process or alternately by crystallizations from methanol. Two different lots of vaccenic acid were prepared and both used in the feeding tests. The identity of the material with vaccenic acid is indicated by the constants obtained, which are summarized in table 2.

The final proof of structure of our vaccenic acid was obtained by  $\text{KMnO}_4$  oxidation of its methyl ester in acetone. The monocarboxylic acids resulting from the oxidation were found to be the expected heptanoic acid, together with the further degradation product, hexanoic acid (Markley, '47); they were redistilled and identified by their boiling points and neutralization equivalents. The dicarboxylic acid was found to have a neutralization equivalent of 109 (calculated for nonanedicarboxylic acid 108) and a melting point of 96°–100° which was lower than the reported 110°C. for this acid but which was in agreement with that found by Boeseken et al. ('30).

We believe that the slightly low iodine value for our material was in part due to the failure of the Wijs reagent, at least as we used it, since pure elaidic acid was also found to have an iodine value of 88.4 (theor. 89.9). Boeseken et al. ('30) reported 88.1 as an iodine value for vaccenic acid.

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# THE GLYCINE REQUIREMENT OF YOUNG POULTS

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## ONE FIGURE

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The need for glycine in the diet of the young chick has been shown by feeding rations in which the amino acids were largely supplied by casein (Almquist et al., '40; Almquist and Mecchi, '40, '42; Hegsted et al., '41), and by feeding rations containing mixtures of amino acids (Almquist and Grau, '44; Hegsted, '44). Jukes et al. ('47) observed that poults grew at a reduced rate when gelatin was replaced by casein in a purified ration in which casein was the only other source of protein, and suggested that the effect of gelatin was due to the glycine which it contained. However, Kratzer et al. ('47) found that casein contains less arginine than is required for the optimum growth of young poults, which suggests that at least some of the growth reduction observed in poults by Jukes et al. may have been due to the deficiency of arginine.

As part of a research into the amino acid requirements of poults, the dietary requirement of young turkeys was investigated by use of a purified ration in which the amino acids were supplied by casein, arginine, cystine and varying levels of glycine.

## EXPERIMENTAL

Day-old poults were fed a practical starting ration for about 6 to 8 days before they were given an adequate purified ration



to accustom them to such a diet. The adequate purified ration contained dried brewers' yeast, casein, gelatin, bone meal, salt, choline chloride, fish oil, soybean oil and cornstarch. After being fed the purified ration for 3 or 4 days the poults were divided into groups of 6 or 7, on the basis of body weight, and were fed the experimental rations. The poults were housed in electrically heated batteries with wire floors. The trials were continued for from 10 to 16 days.

The basal ration contained the following ingredients in grams: washed casein, 28; calcium gluconate, 5; cellulose<sup>1</sup>, 5; salt mixture, 5; crude soybean oil, 3; tri-calcium phosphate, 2; vitamin mixture, 2; dicalcium phosphate, 1.5; L-arginine monohydrochloride, 0.6; fish oil (1000 A-400 D), 0.5; choline chloride, 0.3; L-cystine, 0.2; inositol, 0.1; cholic acid, 0.1; and sufficient cornstarch to make a total of 100 gm. The vitamin mixture had the following composition in milligrams: thiamine, 0.5; riboflavin, 0.5; pyridoxine, 0.4; calcium pantothenate, 1; 2-methyl-1,4-naphthoquinone, 1; niacin, 10; dl-alpha tocopherol acetate, 1; pteroylglutamic acid,<sup>2</sup> 0.1; and biotin,<sup>3</sup> 0.01. The composition of the salt mixture in grams was: sodium chloride, 1; dipotassium phosphate, 0.5; magnesium sulfate, 0.3; potassium chloride, 0.3; sodium silicate, 0.2; manganese sulfate, 0.03; aluminum sulfate, 0.025; ferric oxide, 0.02; copper sulfate, 0.005; zinc sulfate, 0.005; cobalt acetate, 0.002; and potassium iodide, 0.001. Glycine was added at the expense of starch.

Four experiments were completed in which the levels of added glycine varied up to 2% and there were 7 groups which were unsupplemented. Only 3 poults died in all the tests; these losses were apparently not due to the diets.

<sup>1</sup> Cellu flour.

<sup>2</sup> The pteroylglutamic acid was kindly supplied by Lederle Laboratories Inc., Pearl River, N. Y., through the courtesy of Dr. T. H. Jukes.

<sup>3</sup> Synthetic biotin was kindly supplied by Merck and Company, Rahway, N. J., through the courtesy of Dr. D. F. Green.

## RESULTS AND DISCUSSION

The results of the 4 experiments are shown in figure 1. The rates of growth expressed as per cent gain per day<sup>4</sup> varied with the level of added glycine in the ration. As a basis for comparison, the rates of gain of poult fed stock mash in experiments 2, 3 and 4 were 6.9, 7.3 and 6.1% gain per day, respectively.

The growth of poult in general increased slightly as the level of glycine increased until approximately 0.75% glycine was fed. As the level of glycine increased beyond 1.5%, the gains in weight showed a gradual decline. It is evident from the averages shown in figure 1 that the poult in different trials grew at different rates. This was probably due to differences in the poult from different hatches and to slight variations in environmental conditions which we were not able to control. Poult and conditions within a given experiment were comparable.

When the various trials are considered, it is at once apparent that there were no differences between poult fed 0.75 to 1.25% of added glycine, since the average differences in the 10-day gains varied from 5.1 gm (trial 4) to 6.1 gm (trial 2). If we now pair the average gains of groups of poult fed the unsupplemented basal ration with the gains of groups fed 0.75 to 1.25% added glycine in the same experiment, 7 pairs of unsupplemented versus supplemented groups result. The average difference in such a comparison was found to be 19.4 gm giving a value for  $t$  of 7.638 which is statistically highly significant ( $t=3.707$ ,  $p=0.01$ , Fisher, '46). There can be no doubt, therefore, that the addition of 0.75 to 1.25% glycine increased the rate of growth.

Although glycine deficiency caused a reduction in growth, the magnitude of the reduction was only slight. This is shown by the fact that the groups receiving no added glycine (fig. 1)

<sup>4</sup> Per cent gain per day =  $\frac{\text{Total gain} \times 100}{\text{Average weight} \times \text{number of days}}$

grew at a rate comparable to those receiving the stock ration (see above). These results suggest that the comparatively large reduction in growth observed by Jukes et al. ('47) when casein was the only source of protein was most probably due to a deficiency of arginine as well as of glycine, since a deficiency of glycine alone would be expected to produce only a slight decrease.

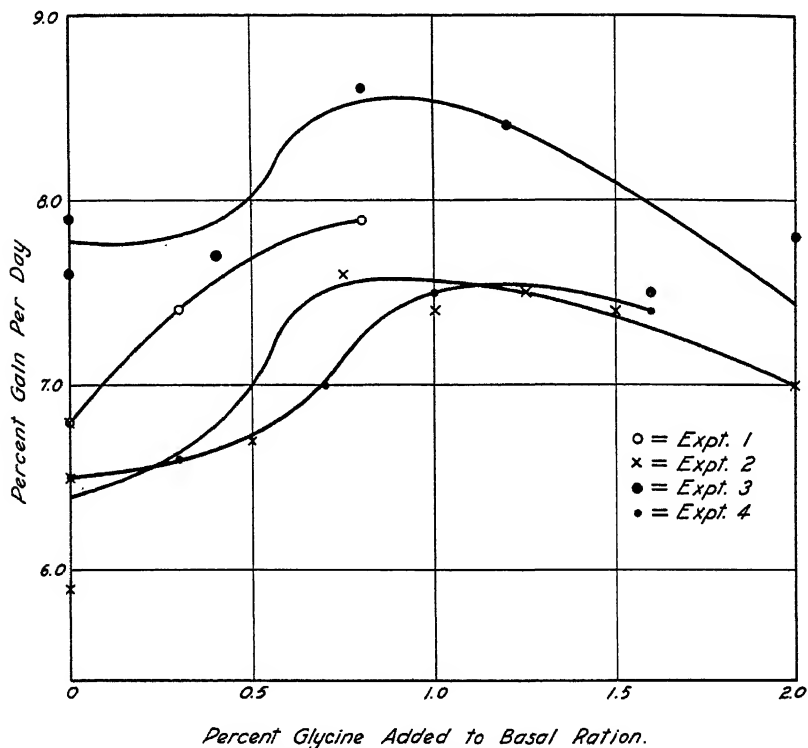


Fig. 1 The relation of the growth of poults to the amount of glycine added to the ration.

Almquist and Mecchi ('42) observed that the growth of chicks was somewhat reduced as the amount of glycine in the ration increased from 1.5 to 2.0%. Patton and Palmer ('36) also noted that glycine fed to laying hens at a rate of 4 gm per day was extremely detrimental to the birds. As

shown in figure 1, the growth of young poults declined as the amount of glycine added to the ration increased above approximately 1.5%. The results of the work on the 2 species indicate that both chicks and poults do not tolerate levels of glycine much in excess of the level which permits optimal growth.

According to Block and Bolling ('45) casein contains approximately 0.5% glycine, hence the basal ration contained 0.12% glycine. The 0.12% plus the 0.75% added glycine needed for optimum growth would place the glycine requirement of the poult at 0.87 or about 0.9% of the ration, a value which is slightly lower than the chick requirement of 1% when the glycine was in combined form, but considerably lower than the level of 1.5% found to be needed by chicks (Almquist and Mecchi, '42) when the glycine was supplied in pure form. The fact that growth of poults in these experiments was depressed less by a deficiency of glycine than was observed in chicks by Almquist and Mecchi ('42) also suggests that poults do not require as much dietary glycine as chicks. The evidence thus suggests that young turkeys possess a greater ability to synthesize glycine than do young chicks.

The lower dietary requirement for glycine of poults as compared with chicks is of special significance in view of the fact that poults require more lysine (Grau et al., '46) and arginine (Kratzer et al., '47), as well as more total protein, than chicks. Evidently the higher protein requirement of poults is not reflected in a higher requirement for all of the indispensable amino acids.

#### SUMMARY

Young poults which were fed a glycine-low ration in which the amino acids were supplied by casein, arginine and cystine grew at a rate comparable to that of poults fed a practical starting ration. Growth was slightly improved by the addition of glycine. Approximately 0.9% of total glycine was the smallest amount which produced optimal growth.

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# GROWTH AND REPRODUCTION OF SWINE ON A PURIFIED DIET<sup>1,2,3</sup>

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## ONE FIGURE

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Growth of swine on purified diets has been reported by several groups of investigators whose experimental objective was the study of vitamin deficiencies. Recently, McRoberts and Hogan ('44) reported on the adequacy of simplified diets and referred to a number of earlier studies of this type.

The experiments presented in this communication were designed to study the effects of a long period of feeding of a purified diet on the development and reproduction of swine.

## EXPERIMENTAL

Pigs of the Jersey-Duroc strain, weaned at 28-31 days, were used. Litter A, born in September, consisted of 6 animals (nos. 25 to 30) and litter B, born in April, comprised 10

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animals (nos. 66 to 75, inclusive). They were distributed in 3 groups, as shown in table 1 and fed as described below.

Group 1, consisting of 2 boars and 3 sows, was fed a purified ration having the following percentage composition: casein,<sup>6</sup> 25; dextrose,<sup>7</sup> 54; hydrogenated cottonseed oil,<sup>8</sup> 13; salt mixture no. 1, U.S.P. XI, 4; bone ash, 2; cod liver oil, 2; and choline chloride, 0.1. The protein content was 20 to 21%.

The members of the vitamin B-complex, available in crystalline form, were compounded in capsules. These were administered daily (except Sunday) by means of a capsule gun which released the capsule in the pharynx. Each capsule contained 2 mg thiamine, 4 mg riboflavin, 20 mg nicotinic acid, 20 mg Ca pantothenate, 20 mg inositol, 10 mg p-aminobenzoic acid and 2 mg pyridoxine. After a body weight of 100 lbs. was reached, the daily dose of these vitamins was doubled. All pigs were given 3 mg alpha-tocopherol by capsule, twice daily. The dosage of the daily vitamin supplement was adopted with the intention of providing the pigs amply with all crystalline B-vitamins. The dosage used, however, was rather arbitrary, since the requirement of pigs has not been established for some of these factors (inositol, p-aminobenzoic acid) nor has it been quantitatively determined for others (nicotinic acid, Ca pantothenate and pyridoxine). The amount of thiamine necessary for maximum growth of pigs maintained on purified diets similar to ours, has been determined as being between 125 and 141  $\mu$ g per 100 gm of carbohydrate and protein (Ellis and Madsen, '44), and the daily requirement for riboflavin appears to be between 1 and 5 mg per 100 lbs. of body weight (Hughes, '40).

For group 2 (3 boars and 1 sow) the same dietary regime was followed as for group 1, except that each animal was fed 10 gm of dried whole liver daily. The dried powder was placed on top of the basal ration and was readily consumed. The ad-

<sup>6</sup> Vitamin-free (Harris).

<sup>7</sup> Cerelease.

<sup>8</sup> Crisco.

TABLE 1  
*Weight gains and food consumption.*

FEEDING REGIME	LITTER, ANIMAL NO. AND SEX	BODY WEIGHT		AFTER 255 DAYS ON EXPERIMENT <sup>1</sup>				TOTAL DAYS ON EX- PERIMENT	FINAL WEIGHT
		Init.	After 140 days on experiment	Weight	Av. daily food con- sumption		Gain per lb. protein consumed		
					lbs.	lbs.			
Group 1, purified diet, crystalline vitamins, 21% protein	A 25 M	15	110	223	2.5	1.56	469	288	
	B 66 M	10	154	271	3.3	1.50	294	273	
	A 26 F	11	96	201	2.4	1.50	469	338	
	B 67 F	13	159 <sup>2</sup>						
	B 68 F	15	160	276	3.5	1.41	294	288	
Group 2, <sup>3</sup> purified diet, crystalline vitamins, dried liver, 21% protein	A 27 M	14	137	257	3.1	1.47	394	350	
	B 69 M	11	166	265	3.9	1.22	294	272	
	B 70 M	9	147 <sup>2</sup>						
	B 72 F	12	161	274	2.8	1.75	294	303	
Group 3, "farm ration," 17% protein	A 29 M	12	159	351	4.9	1.60	469	430	
	B 73 M	12	151	315	4.7	1.60	294	367	
	B 74 M	9	104 <sup>2</sup>						
	A 30 F	13	145	275	4.6	1.29	469	375	
	B 75 F	11	127	260	3.5	1.33	294	303	

<sup>1</sup> 283 days of age.

<sup>2</sup> Sacrificed for histological studies after 140 days of experiment.

<sup>3</sup> In group 2, no. 28 died after 74 days on experiment, and no. 71 was sacrificed for histological studies after 106 days on experiment.



dition of the liver raised the protein content of the ration not more than 1%.

Group 3 served as a control for the animals maintained on the purified diet. It consisted of 3 boars and 2 sows which were fed a hog feed of the open formula type, referred to as the "farm ration." The feed had the following percentage composition: yellow corn, 50; flour middlings, 29; meat scrap (55% protein), 9; soybean oil meal (41% protein), 5; low fiber alfalfa meal, 5; ground limestone, 1.5; and sodium chloride, 0.5. The guaranteed analysis was minimum protein, 17.0%; minimum fat, 3.5%; and maximum fiber, 6.0%. Vitamin D was not supplied because there was sufficient exposure to sunlight.

## RESULTS

### *Growth*

The distribution and disposition of the animals, along with food consumption, are shown in table 1; the growth response is shown in this table and in figure 1. After 140 days of experiment, the animals of litter A fed the "farm ration" showed better gains than their litter mates which received the purified diet with crystalline vitamins. On the diet with the additional supplement of dried liver, the growth response fell between those of the other 2 groups. On the other hand, the animals of litter B on the purified diets grew better than those of litter A and, especially those which received the dried liver supplement, better than those on the "farm ration."

During the first 255 days of the feeding trial (i.e., 283 days of age), all of the animals were housed and fed under comparable conditions. In litter A at the end of this period, boar 29 on the "farm ration" was significantly heavier than boar 27 on the purified diet with crystalline vitamins and the dried liver supplement, and both animals were markedly heavier than boar 25, which received the purified ration and crystalline vitamins. Also, sow 30 on the "farm ration," showed a greater weight than sow 26 on the purified diet with synthetic vitamins. At the end of the 255-day feeding period, boar 73 of

litter B which received the "farm ration," was heavier than litter-mate boars in groups 1 and 2. The sow of litter B on the "farm ration" (no. 75) was slightly lighter in weight than her litter mates on the supplemented purified ration (nos.

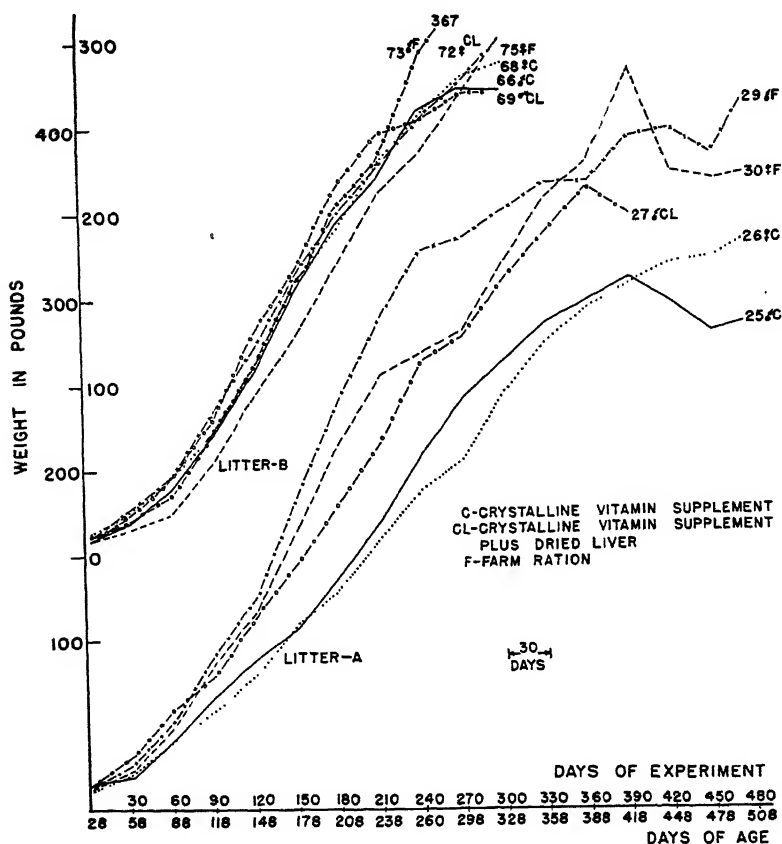


Fig. 1 Growth of swine on the supplemented purified diet and on the "farm ration."

68 and 72) receiving crystalline vitamins and crystalline vitamins and dried liver, respectively, which latter animals had essentially the same body weight.

Except for boar 27, the animals of litter A were continued on the experiment for 214 days longer until they were 497

days of age (fig. 1). In the period following the first 255 days on the experiment, animal 27, receiving liver supplement, and no. 25, receiving only the crystalline vitamin supplement, began to lose weight after 364 and 375 days on the experiment, respectively. On the other hand, no. 26 that was getting the crystalline vitamin supplement and no. 29, fed the "farm ration," continued to grow. Following parturition, the weight of animal 30 remained constant during lactation. At autopsy, pathological conditions were found in certain animals, particularly in those on the purified diet, and these conditions may have been the cause of the decline in weight.

### *Food consumption*

The quantity of purified ration consumed per pound of weight gain during the first 255 days of experiment (table 1, with the exception of animal 69) is slightly less than that of the "farm ration." This was to be expected since the simplified ration contained a higher percentage of protein and less fiber than the "farm ration." For the gain per pound of protein consumed, the range is from 1.22 to 1.75 lbs., but there are no marked differences among the groups, high and low values appearing for each feeding regime. The highest and lowest values for the gain per pound of protein consumed and, with the exception of animal 30, for the feed consumed per pound of gain, are shown by animals 69 and 72 of group 2. Whether this is due either to the difficulty in measuring feed consumption accurately at all times or to high efficiency of utilization by animal 72 and low efficiency by animal 69, is not known.

### *Appearance of animals*

Daily examinations were made, special attention being given to the character of the feces, condition of coat, gait, and condition of the eyes. In the early weeks of the experiment, the feces of all pigs of litter A, even of those on the "farm ration," were soft, and at frequent intervals even diarrheal in character. As the experiment progressed, the feces became well-

formed and only at infrequent intervals were they soft in the case of the animals on the purified diets, and rarely so with the animals on the "farm ration." During the first few months a number of the animals walked with a stiff, jerky gait, at times on tiptoe, or in a fashion resembling goose stepping. Abnormalities in gait appeared earlier and more markedly in animals of litter A than in those of litter B. The animals on the "farm ration" showed lesser signs of abnormal gait. In all animals, however, the abnormal gait gradually disappeared after 6 to 10 months. In general, the coats of the animals were normal although somewhat thin in several animals on the purified diet during the early period of rapid growth. Their hair was often matted together, probably on account of the scattering of their hygroscopic diet. Except for an occasional conjunctival exudate, no abnormal condition was observed in the eyes. All animals behaved quite normally throughout the experiments. However, those on the "farm ration" seemed to be more active and presented, in general, a better appearance than their litter mates on the purified diet.

#### *Autopsy findings*

Only 1 animal (no. 28) died of natural causes during the experiment. Autopsies were performed on all animals except nos. 27 and 74, with the following results:

*Group 1. Purified diet with crystalline vitamins. No. 25, chronic pneumonia of the entire left lung. Bilateral obliterative pleuritis. Chronic focal pericarditis. No. 66, chronic pneumonia of right lower lobe. No. 26, foci of pericardial fibrosis near base of heart over left auricle and ventricle. No. 67, lower lobes of both lungs showed red consolidation. Remainder of lungs normal. Most of small intestine had pin-point-sized red spots. Ulcers varying from 1 mm to about 8 mm in diameter were present in the cecum and scattered along the colon. Some were slightly raised; others extended about 1.5 mm into the lumen and had umbilicated centers. The mucosa of the colon had a granular appearance. No. 68, no pathological findings.*

*Group 2. Purified diet with crystalline vitamins and dried liver.* No. 69, no pathological findings. No. 70, some fatty infiltration in the marrow of the femur. No. 71, no pathological findings. No. 72, some pleural adhesions on the left side. Marked adhesive pericarditis, mainly at the base of the heart.

*Group 3. "Farm ration."* No. 29, no pathological findings. No. 73, no pathological findings, except for dark red color and flabby consistence of the spleen. No. 30, no pathological findings. No. 75, no pathological findings. Pregnant with 6 fetuses.

Of 9 animals maintained on the purified diet, only 3 presented a normal picture on gross examination at autopsy. Evidence of infection was found in 4 animals receiving the supplement of crystalline vitamins, and in 1 receiving the additional supplement of liver. On the other hand, none of the animals maintained on the "farm ration" showed pathological tissue changes, with the exception of a dark and flabby spleen in 1 case.

Therefore, nutrient factors other than those supplied to groups 1 and 2 are apparently necessary to maintain swine in a normal condition on a purified type of diet, in spite of the satisfactory growth performance of some of these animals.

#### MATING AND REPRODUCTION

##### *On the purified diet with synthetic vitamins*

*Boar 25 and sow 26.* Attempts to mate these animals were begun early in July when the animals were 280 days old and had been on the experimental diet for 252 days. On many occasions the boar showed no interest, while at other times he became excited and made futile but short-lasting attempts to service the sow. Finally service was accomplished, but implantation did not take place, since at autopsy 83 days later, fetuses had not formed.

*Boar 66 and sow 68.* Mating attempts were begun at 200 days of age, after the animals had been on the experimental diet for 170 days. Behavior was essentially the same as that of

animals 25 and 26, but mating was not accomplished. Sow 68 was serviced, however, by boar 73 which had been fed the "farm ration." At autopsy, 32 days later, there was no evidence of implantation.

*On the purified diet with synthetic vitamins and dried liver*

*Boar 27.* A sow was not available on this diet. Attempts to mate this animal with sow 26 and with sows maintained on a "farm ration" and pasture were unsuccessful.

*Boar 69 and sow 72.* Attempts to mate these animals were not successful, but sow 72 was serviced by boar 73 of the group which received the "farm ration." At autopsy, 25 days later, there was no sign of implantation.

*On the "farm ration"*

*Boar 29 and sow 30.* These animals were placed on pasture, with free access to the "farm ration," when they were 283 days of age and after they had been on the "farm ration" for 255 days. On November 23, sow 30 farrowed 7 normal pigs and nursed them to weaning at 45 days of age. Therefore, the mating of these animals must have occurred about August 1 or 2, approximately 3 weeks after they were allowed access to pasture.

*Boar 73 and sow 75.* In contrast with animals 29 and 30, these animals did not have access to pasture and received only the "farm ration." They were mated without difficulty when 242 days of age, and 83 days later, at autopsy, the sow was found to be pregnant with 6 fetuses.

DISCUSSION

The question whether a purified diet similar to that used in our experiments and supplemented with crystalline vitamins meets the nutritional requirements of growing pigs has been studied in several laboratories. Wintrobe, Miller, Follis et al. ('42) placed 3 pigs 88 days old on a purified diet supple-

mented with thiamine, riboflavin, niacin, pyridoxine, pantothenic acid and choline. Their animals developed normally and during the test period of 148 days grew at a rate which was only slightly inferior to that obtained on an adequate diet of crude food stuffs. In a later study, the same group of authors (Wintrobe, Stein, Follis and Humphreys, '45) repeated their experiment on 2 pigs 16-22 days old for a period of 120 to 203 days with similar results, although the vitamin supplement lacked niacin but included inositol. Apparently, the experimental period in both studies was too short to allow observations on reproduction.

McRoberts and Hogan ('44) failed to obtain satisfactory growth when they placed pigs at the age of 2 days on a purified diet supplemented with all vitamins available in crystalline form. On the other hand, the addition of relatively large amounts of various extracts from yeast or liver (5% in the diet) enabled the piglets to grow at a satisfactory rate during the experimental period of 20 to 54 days. It is evident from these experiments that pigs — at least during the period of weaning — require nutritional factors other than those provided by the supplement of crystalline vitamins.

The pigs in the present study were placed on a purified diet after weaning at 28 to 31 days of age. The results obtained were comparable to those of Wintrobe, Miller, Follis et al. ('42) and Wintrobe, Stein, Follis and Humphreys ('45), in that satisfactory growth and apparently normal development were obtained on a purified diet supplemented with crystalline vitamins. During the first 3 months of the experiment both sexes grew at least as rapidly on the purified diets as their litter mates on an adequate diet of crude food stuffs. Thereafter, the boars on the "farm ration" gained more weight than those on the purified diet (nos. 29 and 73 vs. nos. 25, 27, 66 and 69). Growth of the sows maintained on the purified diet was, with the exception of no. 26, equal to that obtained for the sows on the "farm ration" over a period of 255 days (cf. nos. 68, 72 and 75). Furthermore, the addition of dried

liver to the purified diet resulted in an improved growth of 1 animal in litter A, but had no effect on the 2 animals in litter B.

At the time this experiment was conducted, biotin was not available in sufficient quantities in crystalline form, and folic acid had not been isolated. According to Cunha, Lindley and Ensminger ('46), a deficiency of biotin in swine results in alopecia, spasticity of the hind legs together with cracks in the feet and dermatosis, but these signs were not noted in the present study. When Lindley and Cunha ('46) fed a purified diet supplemented with crystalline vitamins, except biotin, deficiency symptoms did not appear unless sulfaphthalidine was incorporated in the ration. Therefore, it would appear that the pig obtains its supply of biotin from the intestinal tract in amounts sufficient to prevent the occurrence of these deficiency symptoms. The addition of dried liver to the ration of group 2 of our animals may have provided some biotin and also folic acid, but if present, these factors did not consistently improve growth.

A deficiency disease due to lack of folic acid has not been described in pigs. Signs of deficiency which might be the result of the lack of folic acid in the purified diet could not be noted in this study. However, it remains to be decided whether lack of biotin, or folic acid, or both is responsible for both the increased susceptibility of the animals to infection and their failure in reproduction.

#### SUMMARY

Weanling pigs of 2 litters were maintained, for periods extending to 469 days, (a) on a purified diet supplemented with thiamine, riboflavin, niacin, pyridoxine, pantothenic acid, p-aminobenzoic acid, and choline; (b) on the purified diet with an additional daily supplement of 10 gm of dried liver; and (c) on an adequate commercial hog feed ("farm ration" control).

1. During the first 3 months after weaning, growth on the purified diet was at least equal to that on the "farm ration."



Later, boars on the "farm ration" gained more weight than their litter mates on the purified diet.

2. Appearance and behavior of the animals maintained on the purified diet did not differ appreciably from that of the controls.

3. Feeding of the purified diet resulted in failure to reproduce.

4. The additional feeding of dried liver had no consistent effect on growth of the pigs maintained on the purified diet, and did not induce reproduction.

#### ACKNOWLEDGMENTS

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# PRODUCTION OF FUNCTIONAL AND FATTY CHANGES IN THE LIVER BY A CHRONIC VITAMIN B COMPLEX DEFICIENCY, AND INTERRELATIONSHIPS WITH PROTEIN INTAKE <sup>1</sup>

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FOURTEEN FIGURES

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At present experimental and clinical studies of liver damage are centered chiefly around the effect of methionine and protein intake on the liver. Less attention has been focused on a possible effect of the vitamin B complex. The B vitamins first came into prominence, with respect to liver damage, when they were used in the treatment of alcoholic cirrhosis of the liver (Patek, '37). Since then a number of experimental studies have reported that a deficiency of the vitamin B complex can produce histological liver damage in rats and rabbits (McHenry and Patterson, '44; György, '44). In later work, however, it has been reported that the intake of protein and methionine plays the more important role in experimental hepatic injury. These studies, most of which were histological in character and performed on rats, have been reviewed recently (McHenry and Patterson, '44; György, '44). Of special interest is the report of hepatic cirrhosis in dogs, produced by feeding diets both low in pro-

<sup>1</sup> This study was supported by a grant from Eli Lilly and Company, Indianapolis, Indiana, and the Fluid Research Fund of Yale University School of Medicine.

tein and deficient in one or more of the B vitamins (Fouts, '43).

Few experimental studies have been concerned with the relationship of the vitamin B complex to changes in liver function. Such studies of the vitamin B complex with respect to liver function have been reported in dogs fed a black-tongue producing diet (Rhoads and Miller, '38) and a ration free of vitamin B complex (Drill, Shaffer and Leathem, '43). Similar studies have been made during experimental hyperthyroidism (Drill and Hays, '42; Drill, Shaffer, and Overman, '43). In the studies on the vitamin B complex and thyroid feeding the changes in liver function generally followed the change in caloric intake. Nevertheless, the other studies (Fouts, '43; Rhoads and Miller, '38; Drill, Shaffer and Leathem, '43) lack adequate controls for inanition, so that one cannot judge whether the vitamin deficiency, the concomitant inanition, or both, were factors in producing the hepatic changes. Indeed, mere removal of protein from the diet of dogs will result in dye retention (Hough et al., '43).

The present study was designed to permit observations of possible interrelations between the vitamin B complex and protein as they affect the liver. A *chronic* deficiency of the *whole* vitamin B complex was produced under conditions of controlled protein intake. A *chronic* deficiency was chosen, rather than complete removal of the vitamin B complex from the diet, as being closer to conditions that might be observed clinically. Supplements of whole yeast, analyzed for various B vitamins, were used rather than synthetic vitamin mixtures, because the whole yeast contains more different B vitamins than are usually given in a synthetic vitamin mixture, and may also contain unknown factors. Since clinical recognition of changes in liver function will depend on functional tests and liver biopsies, these techniques rather than determination of hepatic lipids were used to study such changes. The effect of the chronic vitamin B complex deficiency was studied at 2 levels of protein intake and with adequate control of inanition.

## METHODS

Adult mongrel dogs received a synthetic diet containing either 41% or 20% casein.<sup>2</sup> The 41% casein diet is considered to be a high protein diet, whereas the 20% casein ration is regarded as a normal, adequate protein diet. The dogs were allowed the diet *ad libitum* for a 3-hour period each day, after which the food intake was measured. The average food intake per week was calculated as calories consumed per square meter of body surface area per hour, using the formula of Cowgill and Drabkin ('27) for estimating surface area.

In each experiment the dogs were divided into 3 groups. Group I consisted of normal control animals that were used to measure normal fluctuations in food intake. Group II consisted of inanition-control dogs that were limited to the same number of calories, per square meter of surface area, as were consumed by the yeast-deficient dogs during the previous week. The dogs in groups I and II received a supplement of 4 gm of type 3 yeast extract per day,<sup>3</sup> which supplies a normal amount of the B vitamins. Group III consisted of chronic vitamin B-complex deficient dogs receiving a daily supplement of yeast no. 2019.<sup>4</sup> This analyzed yeast was weaker in vitamin content than the type 3 yeast extract, but was of sufficient potency so that the usual supplements fed each day could be weighed accurately. These dogs received 0.1 gm of yeast 2019 per kilo of body weight per day. In a trial experiment with 8 dogs, the chronically yeast-deficient animals were fed 0.2 gm of yeast 2019 per kilo per day. However, this amount of yeast fed over a 25-week period produced only a slight decrease in food intake and no decline in body weight,

<sup>2</sup> The 41% casein diet had the following percentage composition: casein, 41; sucrose, 33.6; lard, 21.5; bone ash, 2.6; and salt mixture (Karr, '20), 1.3, plus 20 drops of *Oleum percomorphum* per kilo of diet. In the 20% casein diet the sucrose was increased to 54.6%, thus keeping the amount of fat constant.

<sup>3</sup> Type 3 yeast extract contained: thiamine, 900  $\mu$ g; riboflavin, 210  $\mu$ g; pantothenic acid, 450  $\mu$ g; nicotinic acid, 4 mg; and pyridoxine, 150  $\mu$ g per gram.

<sup>4</sup> Yeast no. 2019 contained: thiamine, 18-30  $\mu$ g; riboflavin, 65  $\mu$ g; pantothenic acid, 150  $\mu$ g; nicotinic acid, 550  $\mu$ g; pyridoxine, 40  $\mu$ g; choline, 4 mg; inositol, 4 mg; and biotin, 2.5  $\mu$ g per gram.

and liver function and hepatic histology in these animals remained normal. A lower dose of yeast 2019 (0.1 gm per kilo per day) did produce a gradual decline in food intake and was used in the present studies. All dogs received 4 gm of type 3 yeast extract per day during the control period of the study when food intake and measurements of liver function were made.

Changes in hepatic function were studied by means of the bromsulphalein and serum phosphatase tests (Rosenthal and White, '25; Bodansky, '37). A blood sample was taken 30 minutes after the injection of 5 mg of dye per kilo of body weight. The concentration of dye present in the sample was divided by  $2\frac{1}{2}$ , to return it to the scale of standards devised for the original 2 mg dose. A dye retention greater than 6% and serum phosphatase values above 5 units were considered abnormal. Liver biopsies and terminal liver sections were fixed in Bouin's solution and stained in hematoxylin-eosin. Tissue fixed in 10% formalin was stained for fat with Sudan III.

## RESULTS

### *Experiment 1*

#### *High protein diet (41% casein) with minimal yeast supplement*

All animals received the 41% casein diet and, following a control period of 5 weeks, 3 dogs were fed 0.1 gm of yeast 2019 per kilo per day (group 3) to produce a chronic deficiency of the vitamin B complex. Three normal animals (group 1) and 2 inanition controls (group 2) continued to receive 4 gm of type 3 yeast per day.

The dogs receiving the low intake of B vitamins showed a decrease in food intake during the fourth week of the study. By the twenty-fifth week the food intake was only one-third that of the normal dogs (fig. 1). Of the deficient animals, dog no. 1 died during the fourteenth week and dog no. 2 during the thirty-ninth week of the experiment. No change in liver function or histology was noted in the dogs. Renal lesions were

absent in the first dog, but the animal dying during the thirty-ninth week showed kidney lesions in the glomeruli and the tubules. The epithelial cells of the tuft and Bowman's capsule were swollen; proliferation with crescent formation was present; and there was exudate in the glomerular space. In the

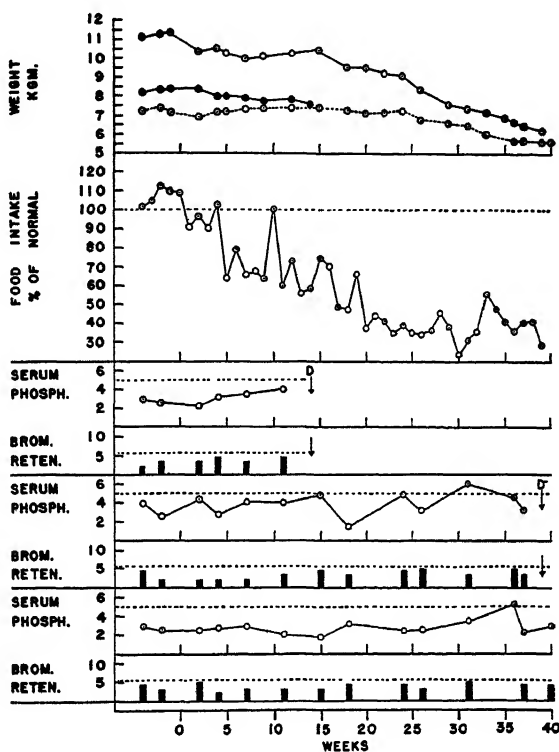


Fig. 1 Effect of a chronic deficiency of the vitamin B complex on liver function and food intake, with a high casein (41%) diet. The food intake represents the average of the 3 dogs receiving 0.1 gm/kg/day of yeast 2019. The liver function tests for dogs 1, 2 and 3 are shown in a descending order. Compare with figures 2, 3 and 4, with dogs receiving the same yeast intake, but a normal (20%) casein diet.

proximal convoluted tubules desquamation, cellular casts, and regenerating tubular epithelium were observed. The adrenal glands were normal in both of these animals. Dog no. 3 was continued on the low intake of yeast 2019 until the fiftieth

week when he was sacrificed. Sections of the liver, kidney and adrenals were normal in this animal.

During this study neither the dogs receiving the low yeast intake nor the inanition controls showed any increased retention of bromosulphalein or serum phosphatase, and sections of the liver were entirely normal.

### *Experiment 2*

#### *Normal protein diet (20% casein) with minimal yeast supplement*

The 8 animals in this study received the 20% casein diet and, following a control period of 5 weeks, were divided into 3 groups as in experiment 1. The 3 vitamin B-complex deficient dogs received 0.1 gm of yeast 2019 per kilo of body weight per day. The 3 normal animals and the 2 inanition controls continued to receive 4 gm of type 3 yeast per day.

*Dogs with subnormal yeast intake.* All 3 animals (group 3) receiving yeast 2019 showed a decrease in food intake. Dog no. 9 developed a marked and rapid decrease in food intake, so that on the one hundredth day he vomited, showed signs of toxemia, and soon was unable to stand. The same day he was given an injection of a mixture of B vitamins with the following composition: thiamine, pyridoxine and riboflavin, 300  $\mu$ g of each; pantothenic acid, 1.5 mg; nicotinic acid, 7.5 mg; inositol, 15 mg; and choline, 75 mg. The dog recovered completely the next day, but during succeeding weeks his food intake increased, and remained at a much higher level than that observed in the other 2 animals (figs. 2, 3 and 4).

During the first 27 weeks of the experiment dogs 10 and 11 showed definite increases in bromsulphalein retention, and dog 9 showed an increase in serum phosphatase (figs. 2, 3 and 4). Similar changes in the functional tests did not occur in the previous experiment in which the dogs received a 41% casein diet. At the beginning of the twenty-seventh week the daily supplement of yeast was reduced by one-half, and produced a further increase in dye retention and serum phosphatase

(figs. 2, 3 and 4). In dog no. 11 serum phosphatase did not increase significantly above normal. A liver biopsy taken during the thirty-second week showed large fat droplets generally distributed throughout the lobule, but with some tendency to central accumulation.

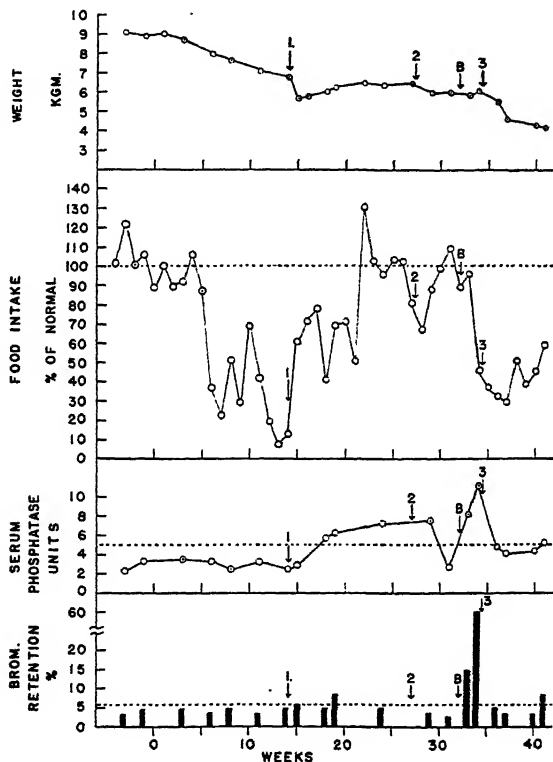


Fig. 2 Effect of a chronic deficiency of the vitamin B complex on liver function and food intake with a normal (20%) casein diet. Data on dog no. 9, receiving 0.1 gm/kg/day of yeast 2019, started at zero time. At 1, an injection of B vitamins was given (see text). At 2, the yeast intake was reduced by one-half. B indicates liver biopsy. At 3, daily supplements of yeast extract were started and total caloric intake was restricted.

Dog 10 showed a rapid decline in food intake during the thirty-third week and died on the two hundred and thirty-first day of the study. For 2 days before death he was given injections of crystalline B vitamins but would not eat during



that time. At the end of the thirty-third week the other 2 deficient dogs were treated with 4 gm of type 3 yeast extract per day. During this period the food intake was limited to 22 cal. per square meter per hour, so that caloric or protein intake would not increase. This feature is represented in figures 2 and 3 as the per cent of food voluntarily consumed by the normal control dogs during this time. Dog no. 9 still

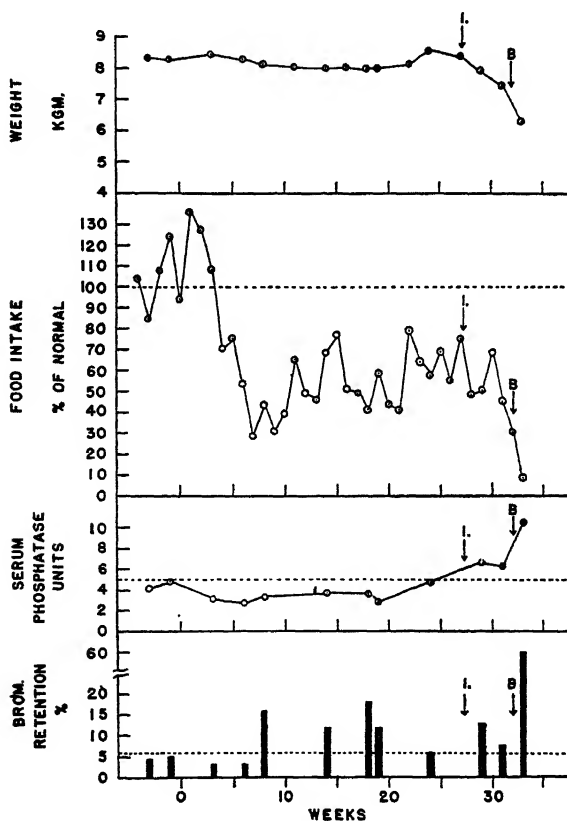


Fig. 3 Effect of a chronic deficiency of the vitamin B complex on liver function and food intake, with a normal (20%) casein diet. Data on dog no. 10, receiving 0.1 gm/kg/day of yeast 2019, started at zero time. At 1, the yeast intake was reduced by one-half. B indicates liver biopsy. After the last functional test in the figure, therapy with B vitamins was started, but the dog died before further functional tests were made (see text).

lost weight on this food allowance so that during the thirty-seventh week he was given 30 cal. per square meter per hour. The dye retention and serum phosphatase of both dogs returned to normal. When these animals were sacrificed during

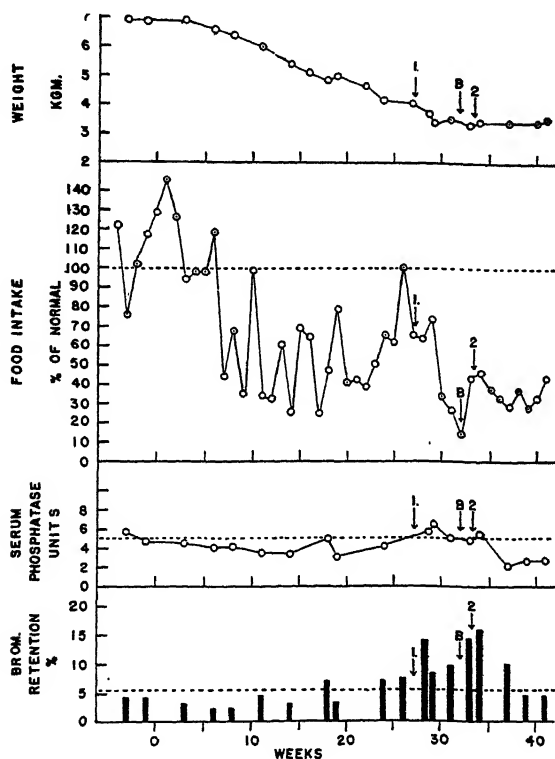


Fig. 4 Effect of a chronic deficiency of the vitamin B complex on liver function and food intake, with a normal (20%) casein diet. Data on dog no. 11, receiving 0.1 gm/kg/day of yeast 2019, started at zero time. At 1, the yeast intake was reduced by one-half, B indicates liver biopsy. At 2, daily supplements of yeast extract were started and the total caloric intake was restricted. Compare with figure 1.

the forty-second week the livers were found to be normal histologically (plate 1).

*Inanition control dogs.* These dogs (nos. 12 and 13) did not show any significant changes in liver function and their

hepatic histology remained normal. On 3 occasions during the study the bromsulphalein retention was slightly above normal, but the change was not consistent and was under 10% (fig. 5). On 5 occasions the serum phosphatase was over 5 units but not above 6 units. Liver biopsies during the thirty-second week and terminal studies of the liver during the forty-second week showed a normal histology. When the food intake of the

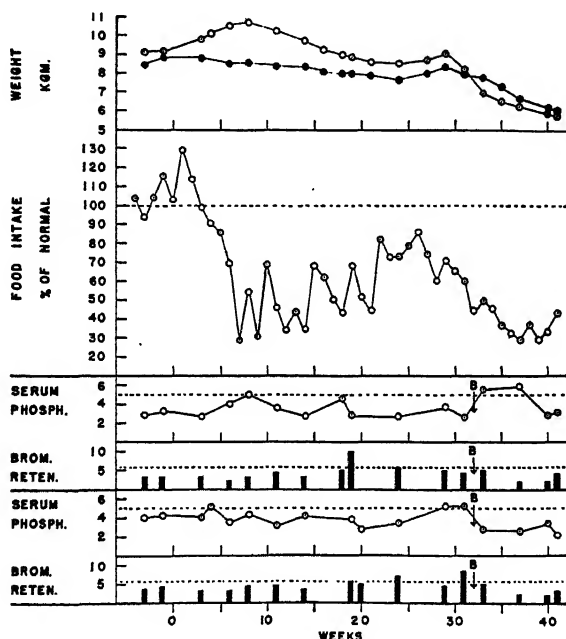


Fig. 5 Effect of inanition on liver function, with a normal (20%) casein diet, and with adequate supplements of yeast extract. B indicates liver biopsy (dogs nos. 12 and 13).

deficient dogs was restricted during the period of yeast therapy, the inanition controls were similarly limited to 22 cal. per square meter per hour.

*Normal control dogs.* The weekly average caloric intake for these 3 dogs (nos. 14, 15 and 16) is shown in figure 6. The bromsulphalein retention remained consistently below 6% in all 3 dogs with only 1 exception (fig. 6). On 5 occasions the

serum phosphatase was above 5 units, the highest value being 5.8 units. Liver biopsies during the thirty-second week and terminal studies of the liver during the forty-second week showed normal hepatic histology.

*Kidney and adrenal glands.* The 3 dogs on the low yeast intake developed renal changes consisting of proliferation of

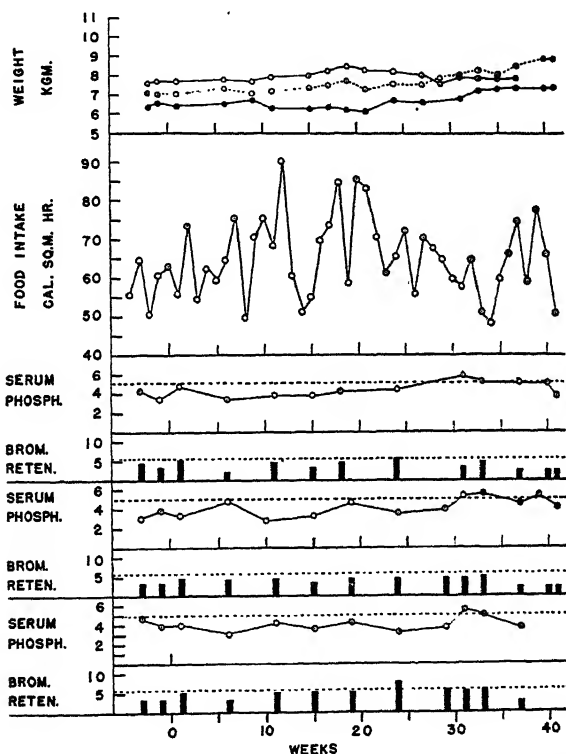


Fig. 6 Food intake and liver function tests of 3 normal control dogs (nos. 14, 15 and 16) with a normal (20%) casein diet.

the epithelial cells of the glomerular tuft and Bowman's capsule with crescent formation. Exudate was also present in the glomerular space. These changes were present in about 50% of the glomeruli and were most prominent in dog no. 9. This animal also had focal scars and periglomerular fibrosis, indicative of spontaneous healed pyelonephritis. None of the

above changes were observed in either the inanition-control dogs or in the normal control animals. There was no change in the adrenal tissue stained with hematoxylin-eosin, but in Sudan III preparations dog no. 10 showed depletion of fat in the fascicular and reticular layers. One of the inanition control dogs also had depletion of fat in the outer fascicular layer.

#### DISCUSSION

In this investigation it was found that a chronic deficiency of the vitamin B complex in dogs may produce functional and histological hepatic changes, depending on protein intake. When each group of dogs received the same low amount of yeast, it was noted that 3 dogs fed a 20% casein diet developed changes in liver function before the twenty-seventh week of study, whereas 3 animals fed a 41% casein diet did not show such a change. Even though the animals received the higher protein diet and low yeast supplements for as long as 50 weeks, no increase in serum phosphatase or dye retention developed. It is evident that a subnormal amount of the vitamin B complex can produce functional changes when a normal (20%) casein diet is fed, but cannot do so when there is a high protein intake (41% casein). That such hepatic dysfunction on the 20% casein diet is not due to inanition can be seen by the lack of changes in the inanition-control dogs that received a normal amount of yeast. The hepatic changes on the 20% casein diet, therefore, must be due to the chronic deficiency in vitamin B complex. However, the fact that they did not develop with the high protein diet is evidence indicating some interrelationship of action, in which either the vitamin B complex or the protein level of the diet is the deciding factor. This relationship may probably be explained by the fact that a low protein diet will reduce the stores of some of the B vitamins in the liver (Wright and Skeggs, '46; Sarett and Perlzweig, '43; Czaczkes and Guggenheim, '46).

When the yeast supplement of the deficient dogs fed the 20% casein was reduced, there was a further change in either the bromsulphalein or serum phosphatase tests. Liver biopsies

were then performed on all animals in this group, but only the yeast-deficient animals showed fatty changes. No hepatic necrosis was observed. The animals were then treated with yeast extract in the same amounts that the normal dogs and inanition controls were receiving. To prevent any increase in protein intake during this period both the vitamin-deficient group and the inanition-controls were given only limited amounts of food. Under such conditions the liver function tests returned to normal and the liver was free of its previous fatty change.

It is of interest that the dogs with functional hepatic changes showed only large fat droplets in the liver and no necrosis. This is in accord with the recent studies of Hims-worth and Glynn ('44a, b). The observations of Gillman and Gillman ('45a, b) from South Africa are also pertinent. They studied the fatty changes in liver biopsy specimens from pellagrins and noted that therapy with riboflavin and nicotinamide increased the liver damage. They also mentioned that the hepatic picture became worse on treatment with brewers' yeast, nicotinic acid and riboflavin. No mention was made of therapy with brewers' yeast alone. When they tried liver extract rich in the fraction G of Cohn, they obtained a slow removal of fat from the liver. However, the most rapid disappearance of hepatic fat was produced by treatment with dried hog stomach (ventriculin). Methionine, in 2 cases, was without effect on the liver fat (Gillman, Gillman and Brenner, '45). These clinical reports also point to the fact that substances other than protein or methionine have a profound effect on at least 1 type of fatty liver. This finding, in general, is in agreement with our results on the relationship of protein intake and a chronic deficiency of the vitamin B complex with respect to functional and histological changes in the liver. This conclusion is not to be interpreted as denying the important role of protein and related substances in liver metabolism, but to point out under controlled conditions the as yet little known action of other substances.

## SUMMARY

1. A *chronic* deficiency of vitamin B complex in dogs fed a high protein (41% casein) diet produced a decrease in voluntary food intake, but liver function tests and hepatic histology remained normal.

2. A similar *chronic* deficiency of vitamin B complex in dogs on a normal (20% casein) diet produced changes in serum phosphatase and bromsulphalein retention. These changes were not present in inanition-control dogs, fed a similar diet plus an adequate amount of vitamin B complex in the form of yeast. A further reduction of the yeast supplement of the chronically deficient animals produced further changes in the functional tests. Liver biopsies showed the presence of large fat droplets but no necrosis. Inanition-controls showed neither functional nor histological changes.

3. The chronically deficient animals were treated with yeast extract, which restored the liver function to normal and produced a disappearance of the large fat droplets in the liver, even though the protein and caloric intakes were restricted during the period of therapy.

4. It is evident that an interrelationship exists between protein intake and a chronic deficiency of the vitamin B complex. Indeed, under conditions of controlled inanition a chronic deficiency of the complex can be shown to play a definite role in producing liver injury.

## ACKNOWLEDGMENTS

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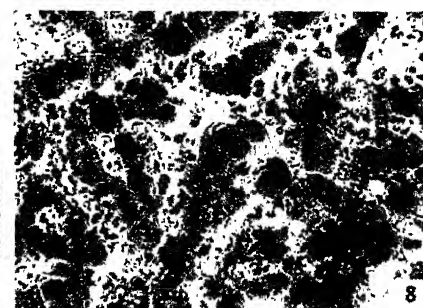
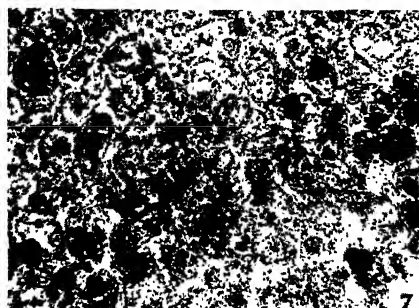
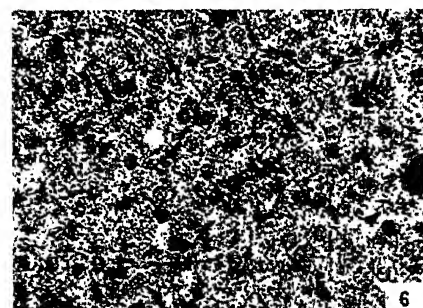
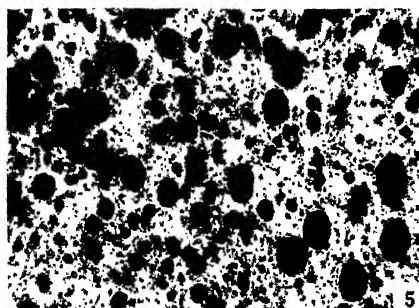
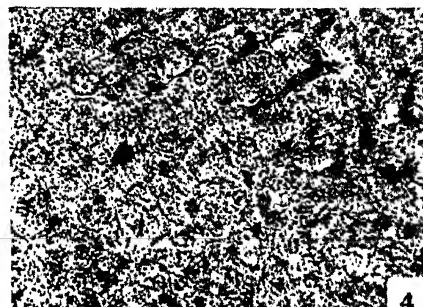
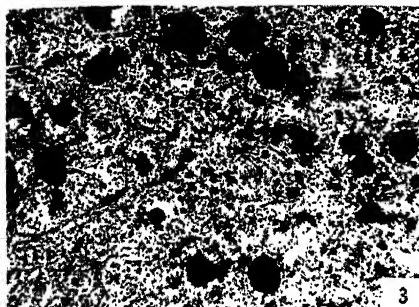
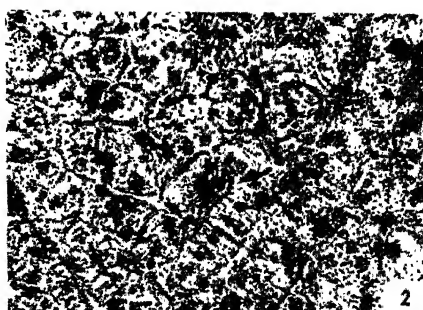
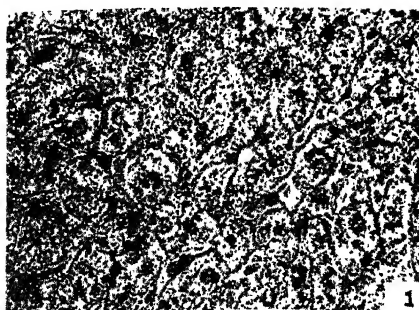
## PLATE 1

### EXPLANATION OF FIGURES

Fatty change in the liver resulting from a chronic deficiency of the vitamin B complex. Tissues fixed in 10% formalin, stained with Sudan III, and photographed through a red filter.  $\times 290$ .

- 1 Dog no. 12, inanition control, liver biopsy, thirty-second week.
- 2 Dog no. 15, normal control, liver biopsy, thirty-second week.
- 3 Dog no. 9, chronic deficiency of vitamin B complex, liver biopsy, thirty-second week.
- 4 Dog no. 9, forty-second week, after treatment with yeast extract.
- 5 Dog no. 11, chronic deficiency of vitamin B complex, liver biopsy, thirty-second week.
- 6 Dog no. 11, forty-second week, after treatment with yeast extract.
- 7 Dog no. 10, chronic deficiency of vitamin B complex liver biopsy, thirty-second week.
- 8 Dog no. 10, forty-second week, died 2 days after treatment with B vitamins was started.

Figures 1 and 2 are representative of the other inanition control and normal dogs in the experiment.





# PANTOTHENIC ACID IN THE NUTRITION OF THE HEN

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## TWO FIGURES

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Pantothenic acid has been shown to be necessary for reproduction in the hen. Gillis, Heuser and Norris ('42) found that when hens were fed a diet of ordinary feedstuffs heated to destroy pantothenic acid their eggs failed to hatch unless this factor was supplied. Studies have also been made of the quantitative pantothenic acid requirement of hens fed a heated diet (Gillis, Heuser and Norris, '43). During the course of these studies, however, it became evident that optimum hatchability could not be obtained with such a basal diet even when it was supplemented with adequate amounts of pantothenic acid. Under these conditions the requirement appeared to lie between 1200 and 1700  $\mu\text{g}$  per 100 gm of diet. When these results were published, it was emphasized that the requirement for pantothenic acid may have been augmented by the deficiency of some other factor or factors in the heated diet. In the absence of other experimental data, however, these results have sometimes been used as a basis for making recommendations concerning the pantothenic acid content of breeder diets for hens.

In an attempt to formulate diets composed of natural feeds without heat treatment, ingredients were assayed and selected on the basis of a low pantothenic acid content. It was

not possible, however, to formulate a satisfactory diet containing less than 500  $\mu$ g of pantothenic acid per 100 gm of diet. The results of limited trials with this type of diet suggested a requirement of between 800 and 1000  $\mu$ g of pantothenic acid per 100 gm of diet for satisfactory hatchability (unpublished results).

In view of the need for more exact information on the requirements of the hen for pantothenic acid, further studies of this problem were undertaken. For this purpose the heated diet of natural feeding-stuffs was replaced by one consisting chiefly of purified ingredients supplemented with the necessary vitamins and minerals. The results reported in this paper include observations on the requirements for maintenance, egg production, and hatchability as well as the effects of different dietary levels of pantothenic acid on the amount of this factor in the hen's blood and eggs, and the effect on the viability of the progeny.

#### EXPERIMENTAL PROCEDURE

The work described in this report consists of 2 experiments conducted during consecutive years. White Leghorn hens in their first year of production were used as experimental animals in both studies. Before beginning each experiment the hatchability and egg-laying capacity of the hens were determined while they were maintained on a commercial breeder diet. The experimental hens were then distributed on the basis of hatchability, egg production, body weight, and general physical condition in order to insure the greatest possible uniformity between the lots in each experiment.

The hens were housed in wire-floored pens with feed and water supplied ad libitum. In the first experiment 6 lots of 8 hens and 1 male each were maintained on the experimental diets for 10 weeks. In the second experiment, which was carried on for a period of 15 weeks, 6 lots of 10 hens and 2 males each were used. In both experiments the males were rotated frequently among the different lots in order to elimi-

nate differences in breeding capacity. Individual records of production and hatchability were kept throughout each experiment. Eggs were incubated weekly and fertility and embryonic mortality were determined by candling on the seventh, fourteenth, and eighteenth days of incubation. At the time of the last candling the eggs were transferred to pedigree hatching trays.

During the second experiment, observations were made also on the effects of different dietary levels of pantothenic acid on the amount of this factor in the hen's blood and eggs. Eggs and blood from all lots were analyzed for pantothenic acid at intervals as described below. During this experiment the growth and livability of chicks produced by hens receiving different levels of pantothenic acid were also studied.

The basal diet used in these experiments had the following percentage composition: corn starch 59, crude casein 15, gelatin 5, fish meal 4, cellophane 4, soybean oil 3, cod liver oil 0.5, oyster shells 2, limestone 2, dicalcium phosphate 3, salt mixture 2<sup>1</sup> and vitamin supplements 0.5.<sup>2</sup> Fish meal was included in the diet to furnish the factor in liver paste which was previously found to be necessary for hatchability (Gillis et al., '42), and which subsequent work by a number of investigators has indicated to be present in fish meal and other animal proteins. Microbiological assay of this diet, using *Lactobacillus arabinosus* as the test organism, showed it to contain approximately 1.5  $\mu$ g of pantothenic acid per gram (pantothenic acid as used here refers to calcium pantothenate, dextrorotatory). The different levels of supplemental calcium pantothenate, in aqueous solution, were added to the basal mixture at weekly intervals to prevent destruction in storage.

<sup>1</sup> Salt mixture per 100 pounds:  $K_2HPO_4$  420 gm, NaCl 275 gm,  $MgSO_4 \cdot 7H_2O$  185 gm,  $FeSO_4 \cdot 7H_2O$  19 gm,  $MnSO_4 \cdot 4H_2O$  5 gm, KI 1.5 gm,  $ZnCl_2$  1 gm,  $CuSO_4 \cdot 5H_2O$  1 gm,  $CoCl_2 \cdot 6H_2O$  0.5 gm.

<sup>2</sup> Vitamins per 100 pounds: thiamine 0.15 gm, riboflavin 0.22 gm, pyridoxine 0.22 gm, niacin 1 gm, folic acid 0.1 gm, vitamin K 20 mg, biotin 7 mg, inositol 40 gm, wheat germ oil 70 gm, choline 100 gm.

## RESULTS

*Hatchability, egg production and maintenance*

The results on hatchability in both the first and second experiments are given in table 1. In the first experiment the requirement for optimum hatchability was between 750 and 1000  $\mu\text{g}$  pantothenic acid per 100 gm of diet. However, in the second experiment, 650  $\mu\text{g}$  resulted in as good hatchability as any of the higher levels. These results indicate a considerable genetic variation among hens in their pantothenic acid

TABLE 1

*Average hatchability of fertile eggs from hens receiving different levels of pantothenic acid ( $\mu\text{g}$  pantothenic acid per 100 gm of diet).*

Weeks inclusive	EXPERIMENT 1					
	150 $\mu\text{g}$	250 $\mu\text{g}$	500 $\mu\text{g}$	750 $\mu\text{g}$	1000 $\mu\text{g}$	1500 $\mu\text{g}$
	%	%	%	%	%	%
1st-3rd	93	80	90	93	92	82
4th-7th	38	57	72	79	87	80
8th-10th	0	11	52	56	85	79
	EXPERIMENT 2					
	150 $\mu\text{g}$	350 $\mu\text{g}$	650 $\mu\text{g}$	800 $\mu\text{g}$	950 $\mu\text{g}$	1100 $\mu\text{g}$
	%	%	%	%	%	%
1st-3rd	95	87	92	93	95	95
4th-7th	20	84	91	91	96	91
8th-10th	6 <sup>1</sup>	75	91	91	94	92
11th-15th	2	76	95	92	92	92

<sup>1</sup> 10th week, zero. <sup>2</sup> Discontinued.

requirement. This is not surprising in view of the wide variation which several investigators have found in the pantothenic acid requirements of young chicks. The lower requirement for the hens in the second experiment can be explained, in part at least, by the degree of selection to which they were subjected. The number of hens with preliminary hatching records available was considerably greater for the second experiment than for the first, resulting in a more rigid selection of the hens for good hatchability.

In both instances the hatchability of eggs from the basal lot receiving 150  $\mu\text{g}$  of pantothenic acid per 100 gm of diet

declined to zero. The complete failure of hatchability on this deficient diet is further evidence of the vital role of pantothenic acid in reproduction. The excellent hatchability obtained when the basal diet was supplemented with adequate pantothenic acid indicates that the diet used is probably adequate in all nutritional factors required by the hen other than pantothenic acid. The results obtained in these experiments involving a purified diet show that the pantothenic acid requirement of hens is considerably lower than that obtained on the diet of heated natural feedstuffs. A deficiency in the heated diet of some other factor required for hatchability undoubtedly operated to increase the requirement for pantothenic acid.

A deficiency of pantothenic acid in the diet of hens did not result in characteristic deformities in chick embryos such as are obtained with a riboflavin deficiency. Although Taylor, Mitchell and Pollack ('41) have reported slightly abnormal or subnormal development in certain chick embryonic tissues and organs, gross inspection of embryos which failed to hatch did not reveal any characteristic syndrome. Poor quality of down, however, was sometimes observed in the embryos which failed to hatch as well as in some of the chicks hatched on the lower levels of pantothenic acid. In these studies it was again impossible to find any support for the claim of Taylor et al. ('41) that pantothenic acid favorably affects embryonic survival during the first 13 days of incubation. Practically all of the mortality in these experiments occurred after the candling on the eighteenth day. Many of the embryos that failed to hatch were alive on the twenty-first day and actually succeeded in pipping the egg shell. Thus, most of the embryos appear to be able to develop even when a pantothenic acid deficiency exists in the egg, but when they are required to break out of the shell, they are unable to perform this task.

The decline in hatchability of fertile eggs did not keep pace with the rate of depletion of the hens and of their eggs in pantothenic acid. This strongly suggests that at least part



of the effect of a pantothenic acid deficiency is due to a disturbance of the reproductive physiology aside from a lowering of the pantothenic acid content of the egg. A longer period is required to bring about this disturbance than is needed to deplete the hen of pantothenic acid which is not stored to any appreciable extent. The administration of pantothenic acid to deficient hens, however, resulted in an immediate resumption of hatchability. This is illustrated by the basal lot in the second experiment. This lot which received the unsupplemented diet declined in hatchability from 97% to zero, and was taken off the experiment after the tenth week. The hens from this lot were divided into 2 equal groups which were fed the basal diet supplemented with calcium pantothenate in

TABLE 2

*Percentage hatchability following administration of pantothenic acid (P.A.) to depleted hens.*

DEPLETION PERIOD		RECOVERY PERIOD			
P.A. per 100 gm diet	Hatchability (10th wk.)	P.A. per 100 gm diet	Hatchability		
			(11th wk.)	(12th wk.)	(13th wk.)
$\mu g$	%	$\mu g$	%	%	%
150	0	800	40	96	93
150	0	1100	71	91	88

sufficient amount to raise the total to 800 and 1100  $\mu g$  per 100 gm of diet, respectively, for the remaining 5 weeks of the experiment. Their immediate recovery of hatchability is shown in table 2.

The effect of different levels of pantothenic acid on the rate of egg production was observed in both experiments. In neither experiment was there a correlation between egg production and the levels of pantothenic acid fed. Although hens were not maintained on the basal diet longer than 10 weeks, no decrease in egg production was observed during this period. During the second experiment, egg production actually increased appreciably during the 10-week period that the hens were maintained on the basal diet. The requirement for egg production, therefore, is considerably less than for hatch-

ability and does not appear to be greater than 150  $\mu$ g of pantothenic acid per 100 gm of diet. It is possible, however, that the requirement might be found to be greater if hens were maintained on such a low level of pantothenic acid for a more protracted period.

The average weight of the hens in all lots in both experiments increased during the experimental period and their general physical condition was good at the end of the feeding trial. The response of the basal lot was as good in this respect as that of any of the lots receiving pantothenic acid supplements, although this lot was not maintained beyond 10 weeks in either case. Mortality was negligible in all lots. The results of these experiments suggest that the maintenance requirement of the adult fowl is not greater than 150  $\mu$ g of pantothenic acid per 100 gm of diet. This is about 25% of the requirement of the rapidly growing chick.

*Effect of level of pantothenic acid in the hen's diet  
on the viability and growth of the chicks  
produced*

Pantothenic acid deficiency does not seriously interfere with the development of the chick embryo until near the time of hatching. As a consequence of the ability of the embryo to develop even when the egg is deficient in pantothenic acid, many chicks actually hatch from eggs containing suboptimum levels of pantothenic acid. Most of the chicks from hens receiving the lower levels of pantothenic acid were weaker when hatched than were chicks from hens receiving more pantothenic acid. This has been a constant observation in experiments with both the heated and purified diets. An optimum allowance of any nutritive factor for breeding animals is one which permits the production of the greatest number of vigorous progeny. Therefore, the requirement of breeding hens for pantothenic acid is not simply for hatchability, but for the production of chicks strong enough to live and make rapid gains when properly fed. It is important that

the newly-hatched chick have sufficient body stores of pantothenic acid to last it until this factor can be assimilated in proper amounts from the food it consumes. Williams et al. ('41) have shown that the tissues of the chick are lower in pantothenic acid content at the time of hatching than during embryonic development or later life.

A study was made to determine the level of pantothenic acid in the hen's diet which results in the lowest mortality and most rapid growth of the progeny. During the eleventh and again during the thirteenth week of the second experiment 20 chicks were selected from each lot (except the basal

TABLE 3

*Effect of pantothenic acid content of the hen's diet on survival and early growth of chicks.<sup>1</sup>*

P.A. PER 100 GM HEN'S DIET	WEIGHTS OF CHICKS AT 4 WEEKS	MORTALITY
<i>μg</i>	<i>gm</i>	<i>%</i>
350	253	50
650	268	0
800	281	0
950	304	0
1100	296	0

<sup>1</sup> Average of 2 experiments — 40 chicks.

which had ceased to hatch) for use in these studies. As nearly as possible an equal number of chicks from each hen was used. The chicks from each lot were weighed, wing-banded, and placed in electrically heated battery brooders when 1 day old. All lots were given the same practical type diet containing approximately 1400  $\mu$ g of pantothenic acid per 100 gm.

The results on growth and mortality of chicks from all lots are given in table 3. Mortality was encountered only in the lot from hens receiving 350  $\mu$ g of pantothenic acid per 100 gm of diet. Both survival and growth response were very poor in this lot, leaving no doubt as to the inadequacy of this level of pantothenic acid for breeding hens. In addition to heavy mortality and retarded growth, feathering was very poor in

this lot at the end of the fourth week. About 50% of the chicks surviving in this group showed signs of a mild dermatosis at some time.

Growth of chicks from the lot receiving 650  $\mu$ g of pantothenic acid was less satisfactory than that of chicks from lots receiving more of this vitamin. A few of the chicks from this lot exhibited some nervous disorders, such as turning the head from side to side or tremors; however, there were no symptoms of dermatosis. It appears that at least 800  $\mu$ g of pantothenic acid in 100 gm of the hen's diet is necessary to produce the most viable chicks capable of making rapid early gains in weight. This conclusion is based on the fact that 800  $\mu$ g prevented all symptoms of deficiency in the chicks and permitted growth which was not significantly below that of chicks from lots receiving more pantothenic acid. The growth of chicks from the lot receiving 650  $\mu$ g was significantly below that of chicks from the 2 lots receiving the highest levels of pantothenic acid.

*Influence of the diet on the pantothenic acid content  
of the hen's blood and eggs*

The effect of the dietary level of pantothenic acid on the amount of this factor in the hen's blood and eggs, as well as on the rate of depletion was studied during the second experiment. Microbiological assays for pantothenic acid were conducted on both blood and eggs from all lots at the beginning of the experiments and at intervals thereafter. The assay procedure was a modification of the method of Skeggs and Wright ('44). Since preliminary tests showed that enzymatic treatment of these materials resulted in no increase in pantothenic acid content, this step was eliminated in the assays reported here.

One-milliliter samples of blood were withdrawn from the wing veins of each hen for assay purposes. The samples of whole blood from 3 or 4 hens were then combined, diluted with water, autoclaved, filtered, and suitable aliquots of the filtrate

used for the assays. The procedure with eggs was as follows: 3 or 4 eggs were broken into a graduated cylinder, weighed, and the volume adjusted with distilled water to 1 ml per gram of whole egg. The whole eggs were then homogenized in a Waring Blendor, and an aliquot of the homogenous material diluted with distilled water, autoclaved, and filtered. The filtrate was used for the assay.

The pantothenic acid content of the diet was quickly reflected in the amount of this factor found in the blood. At

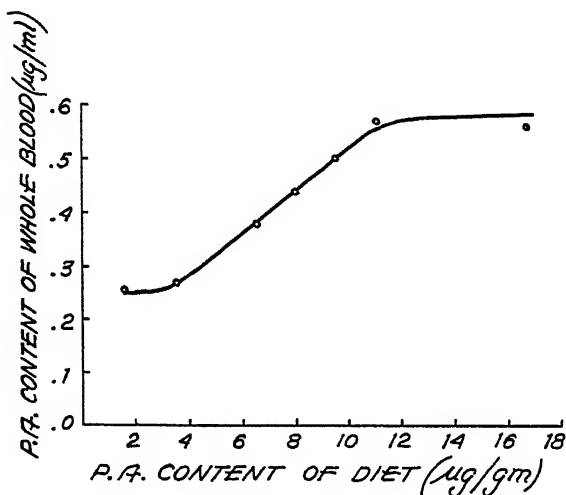


Fig. 1 The effect of different dietary levels of pantothenic acid on the amount of this factor in hen's blood.

the beginning of this experiment, the pantothenic acid content per milliliter of whole blood was approximately 0.56  $\mu\text{g}$  for all lots. The amount of pantothenic acid in the blood became stationary within 3 weeks after the hens were placed on the experimental diet. The relationship between the pantothenic acid content of the diet and that of the blood is represented graphically in figure 1. The response of the blood to different levels of dietary pantothenic acid is a straight-line relationship within the limits of about 350–1100  $\mu\text{g}$  per 100 gm of diet. Above or below these values, however, the curve flattens out. This sug-

gests that the animal exerts some control over the amount of this vitamin in the blood, probably through control of its excretion as certain limits are approached. Approximately 1100  $\mu$ g of pantothenic acid were required in this experiment to maintain saturation of the blood with this factor. Since optimum reproductive performance was associated with a level of about 800  $\mu$ g of pantothenic acid, saturation of the blood is not necessarily a criterion of adequate nutrition.

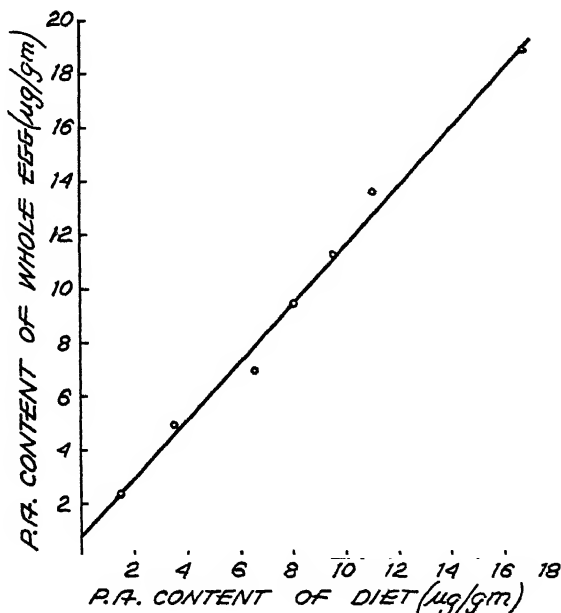


Fig. 2 The effect of different dietary levels of pantothenic acid on the amount of this factor in hen's eggs.

The pantothenic acid content of eggs is likewise immediately affected by the amount of this factor in the diet. The values for eggs did not decrease after the third week. The relationship between the dietary level of pantothenic acid and the amount of this factor in the egg is shown graphically in figure 2. It will be observed that a straight-line relationship exists between the pantothenic acid content of the diet and the eggs over the range of values studied in this experiment. This

relationship is expressed by the equation,  $Y = 1.1 X + 0.7$ , in which Y equals the micrograms of pantothenic acid per gram of whole egg, and X equals the micrograms of pantothenic acid per gram of diet. These values agree fairly well with those reported by Pearson, Melass and Sherwood ('45). In this experiment, optimum results were associated with pantothenic acid levels of about 9.5  $\mu\text{g}$  or more per gram of whole egg.

#### SUMMARY

Studies have been made of the pantothenic acid requirement of hens fed a diet of purified ingredients supplemented with 4% of fish meal. Under these conditions, the requirement for maximum hatchability was found to vary somewhat, depending on the degree to which the hens were selected for hatchability. In 1 experiment, the requirement for hatchability was found to lie between 750 and 1000  $\mu\text{g}$  of pantothenic acid per 100 gm of diet. In a second experiment although 650  $\mu\text{g}$  supported optimum hatchability, it was found that at least 800  $\mu\text{g}$  per 100 gm was necessary in the diet of the hens to promote best growth, and to prevent symptoms of deficiency and mortality in their progeny. It appears from the lower requirement obtained with the purified diet, that a deficiency in the heated diet previously used considerably increased the requirement for pantothenic acid. When hens depleted of pantothenic acid were supplied this vitamin, hatchability of their eggs returned to normal in 1 or 2 weeks.

Embryonic mortality due to pantothenic acid deficiency was found to be confined almost entirely to the last 2 or 3 days of the incubation period. The deficiency did not result in deformities in the embryos. Chicks hatched from hens receiving suboptimal levels of pantothenic acid were generally inferior, however.

Egg production and maintenance of weight were not adversely affected during a 10-week period by a pantothenic acid level as low as 150  $\mu\text{g}$  per 100 gm of diet. Hens maintained for 10 weeks on a diet containing this amount of pantothenic acid actually increased their body weight during 2

different experiments, and in 1 instance increased the rate of egg production.

The pantothenic acid content of the diet is quickly reflected in the amount of this factor found in the blood and eggs. The amount stored in eggs was found to fit the same straight-line equation for all dietary levels studied. The amount of pantothenic acid in the blood is much lower than that for eggs, and tends to remain constant below 350  $\mu\text{g}$  per 100 gm of diet, or above 1100  $\mu\text{g}$ . The minimum pantothenic acid content of whole blood associated with optimum reproductive performance was 0.45  $\mu\text{g}$  per milliliter. For eggs, the corresponding value was 9.5  $\mu\text{g}$  per gram of whole egg.

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# AN ADJUSTABLE CYLINDRICAL CAGE FOR USE IN METABOLISM STUDIES WITH YOUNG PIGS

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## TWO FIGURES

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The problem of adequate caging frequently arises when young pigs are to be employed in metabolism studies, and especially where accurate separation of excreta is an essential. For pigs over 50 pounds live weight the rectangular-type metabolism cage as used by Watson et al. ('43) and by Crampton and Whiting ('43) is very satisfactory and offers few collection difficulties. Pigs from weaning age to 50 or 60 pounds live weight probably resent confinement more than do heavier animals and, being considerably more agile, such young pigs have to be confined very closely when in rectangular cages in order to prevent them from turning.

With these facts in mind it seemed worthwhile to design a cage which would allow maximum freedom of movement and still permit separate collection of excreta from male pigs below about 50 pounds live weight.

## DESIGN

### *General*

The cage consists essentially of a rectangular strip of wire mesh bent into a cylindrical shape to form the walls, and anchored on a heavier wire mesh floor by means of expansion-

<sup>1</sup> The author wishes to acknowledge the assistance of M. J. Brinegar.

type coil springs. In order to confine the animal from above, and yet permit easy adjustment, the top is suspended from the ceiling by light chains at the back and with a chain and hook at the front for use when the top is up. Expansion coil springs also serve to retain the top in position when the cage is closed.

As support for the basic structure described above, a bench large enough to hold 4 cages was constructed. Underneath each cage a central funnel directs the urine to a collection bottle below the bench.

In this type of metabolism cage, feeding is accomplished by placing a suitable trough in the cage until the animal has finished eating.

### *Details of construction*

*Walls.* One piece of  $2' \times 9'$  flattened expanded metal <sup>2a</sup> is bent in the form of a cylinder of about  $3'$  diameter, and provided with 2 hooks or clasps for holding the cylinder at any desired size.

*Top.* This consists of a  $3' \times 3'$  wooden frame of  $2'' \times 2''$  supporting  $\frac{5}{8}''$  iron rods  $3''$  apart. Two pieces of chain suspend the back of the top from the ceiling. Two expansion-type coil springs serve to hold the top in position when the cage is closed.

*Floor.* Four pieces of  $3' \times 4'$  flattened expanded metal <sup>2b</sup> are laid on a  $3' \times 16'$  frame of  $2'' \times 4''$  lumber, with  $\frac{3}{16}'' \times 1\frac{1}{2}''$  strap iron cross-braces at  $12''$  intervals throughout the length of the frame. This frame is provided with angle-iron legs to hold the cage floors  $9''$  above the bench. This permits easy collection of the feces which fall through the floor.

*Bench.* This is  $28''$  high  $\times 40''$  wide  $\times 16'$  long, well-braced and provided with  $2'' \times 4''$  legs at intervals of  $4'$ .

<sup>2</sup> Wheeling Corrugating Company, Buffalo, N. Y.

a. Design no. 9-11,  $1\frac{1}{2}''$  flattened expanded metal (plain).

b. Design no. 9-11,  $\frac{3}{4}''$  flattened expanded metal (plain).

On the bench under each cage, a galvanized metal sheet serves to provide an easily cleaned surface for the fecal collections. A gradual slope to the sheet, and facilities for collecting liquid excreta have proven useful, especially while the cage is being adjusted to the animal for the first few days.

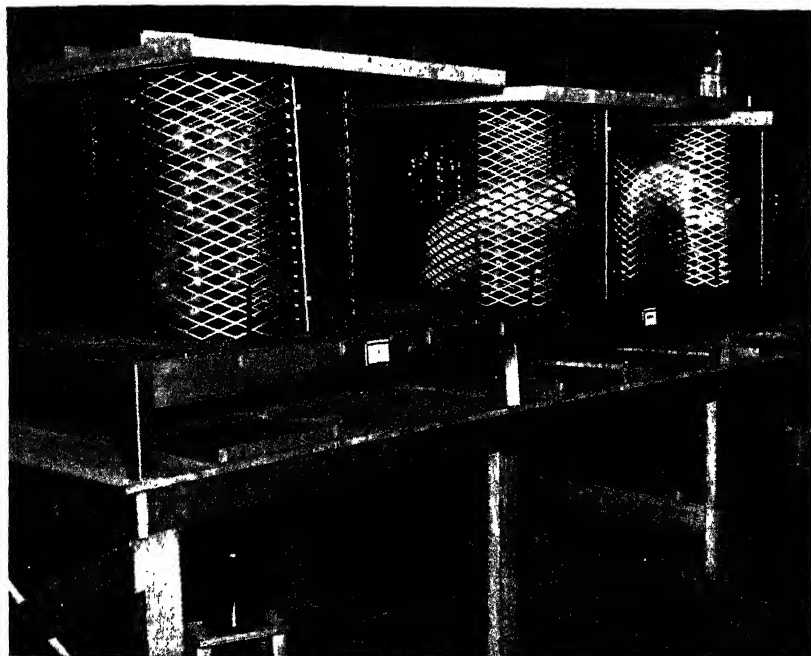


Fig.1 Front view of a battery of cages in use.

*Funnel.* It is 16" in diameter, placed directly under the center of the cage below the floor. It is shallow and has a short stem to permit easy removal. A rubber connection below the bench directs the urine into the bottle. Each funnel possesses a screen and, at the bottom, some coarse glass wool to insure further against contamination.

*Feeder.* The sides are 16" high sloping to 4" high at the front, with a base of 7". Two such sides together with a 9"  $\times$  16" back and a 4"  $\times$  9" front piece enclose a metal,

water-tight, round bottom feed container. A small hook at the back and 2 screws protruding  $\frac{1}{2}$ " down from the bottom adequately anchor the feeder while it is in the cage.



Fig. 2 Close-up view of a pig in a cylindrical cage. The top is up, ready for feeding or handling. The method of controlling the circumference of the cage is shown in the front.

#### DISCUSSION

While these cages have been in use pigs ranging in weight from 20 to 90 pounds have been observed. The cages were ordinarily adjusted to the length of the pig during the preliminary period on a given diet, following which the central funnel system in all cases permitted a highly satisfactory separation of urine and feces. Most well-formed feces pass through the floor, and those which remain are easily removed by raising the wall of the cage.

Since the feeding and collection methods permit no contamination of feces with feed, a high degree of precision can

be expected in balance work. The control of coprophagy has offered no problem. It is most probable that pigs under these conditions will not consume feces except incidentally when eating spilled feed amidst excreta. The feeder described above tends to crowd the pig and does not encourage spilling of feed.

The experience of this laboratory with the adjustable cylindrical cage indicates that it is to be preferred to the rectangular-type cage for use with small male pigs employed in controlled digestion and metabolism studies.

#### SUMMARY

An adjustable cylindrical cage is described for use in metabolism studies with male pigs, particularly those below 50-60 pounds live weight. In the experience of the author, several months' trial has indicated that the central funnel arrangement for urine collection is a practical one, highly efficient in permitting separation and collection of excreta. It allows some freedom of movement. The usual preliminary period allows sufficient time for adjusting the size of the cage to the animal.

Details of construction and illustrations of the cages in use are included.

#### ACKNOWLEDGMENT

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# DENTAL CARIES IN THE COTTON RAT

## X. THE EFFECT OF FLUIDITY OF THE RATION <sup>1</sup>

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In the course of investigations on dental caries in the cotton rat Schweigert and co-workers ('46b) found that this animal was free of carious lesions when fed a diet of liquid whole milk. This was in contrast to the extensive dental decay developed on dry rations high in sucrose or other fermentable carbohydrates. Milk was found to exert a protective action even in the presence of 5 or 10% of fermentable sugar, although caries scores on such rations were not zero as they were on a diet of liquid milk alone (Anderson et al., '47). The higher caries scores when a dry ration was fed seemed to indicate that fluidity of the ration might play an important part in this protection. Studies on various dry and liquid rations were therefore undertaken.

### EXPERIMENTAL

The experimental groups were set up according to the procedure of Shaw and his associates ('44a), using weanling cotton rats (20–25 gm) from our stock colony. Litters were divided among the groups to allow for differences in caries susceptibility (Schweigert et al., '45). The animals were killed

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at the end of the 14-week experimental period and the incidence and extent of carious lesions determined by the method of Shaw and co-workers ('44a, '44b).

Control animals were fed a dry ration known to be highly cariogenic.<sup>2</sup> Dry milk rations were fed as dry whole milk and as dry skim milk plus butter. The latter was prepared by adding butter to non-fat dry milk solids to make a ration of 21.3% butterfat. Both of these rations were supplemented with 4% of 1:20 liver extract, and minerals were added to make the intake of these the same as that of the animals which had been fed liquid milk.<sup>3</sup> Since the minerals were added in solution, these rations were dried after the addition of minerals and butter and subsequently ground in a Wiley mill. This was done merely to produce a ration of even consistency, and no attempt was made to control particle size.

Two other groups of animals received the dried milk rations reconstituted with water to produce equivalent liquid milk rations. To make the composition approximate that of liquid whole milk the rations were made up to 12.5% solids (14.3 gm per 100 ml of water). The respective dry milk rations were each mixed with warm water in a Waring blender and then put through a hand homogenizer. Since the greater bulk of the liquid rations reduced the intake of solids, a further addition of minerals was made to these rations to equalize the consumption of these dietary factors.

A further experimental group was fed the cariogenic control ration 802 suspended in water. The casein was ground to a finer consistency in a ball mill before being mixed with the fat and soluble constituents. This ration was then mixed with water in the same way as the reconstituted dry milk diets above, i.e., on a 12.5% solids basis. Liver extract was added at the usual level of 4% of the solids. This ration was also

<sup>2</sup> This ration (802) had the following percentage composition: sucrose, 67; casein, 24; corn oil, 5; and salts IV, 4. Four per cent of 1:20 liver extract was added at the expense of the entire ration. Adequate quantities of the B vitamins (McIntire et al., '44) were added to the ration, and each rat received 1 drop of halibut liver oil per week.

<sup>3</sup> A daily intake of 1 mg of iron and 0.1 mg of copper and of manganese.

put through the hand homogenizer. The treatment did not produce a true liquid but served to put the more insoluble solids into a fine suspension. All rations were fed *ad libitum*.

### RESULTS

The data on growth rate and caries indices are given in table 1. Experimental animals are paired with their litter-mate controls in each case. The animals from each litter were divided as equally as possible between the control and experimental groups, and if there was an extra animal in either group the average of the other group was weighted accordingly.

Growth rates are given as average weekly gains for the first 6 weeks and for the total 14 weeks on experiment. All

TABLE 1

*The effect of various dry and liquid rations on growth rate and on incidence<sup>1</sup> and extent<sup>2</sup> of carious lesions in the cotton rat.*

RATION	NUMBER OF ANIMALS	AVERAGE WEEKLY GAIN		AVERAGE INCIDENCE	AVERAGE EXTENT
		6 wks.	14 wks.		
		<i>gm</i>	<i>gm</i>		
802 Dry cariogenic control	12	11	7	25	59 +
Dry whole milk mixture	12	11	6	8	13 +
802 Dry cariogenic control	14	10	7	24	57 +
Dry reconstituted whole milk	14	8	5	1	1 +
802 Dry cariogenic control	15	11	8	28	70 +
Dry skim milk plus butter	15	8	7	14	30 +
802 Dry cariogenic control	11	11	8	32	57 +
Dry reconstituted skim milk plus butter	11	7	5	1	1 +
802 Dry cariogenic control	10	11	7	24	60 +
802 Liquid cariogenic control	10	7	5	9	14 +

<sup>1</sup> The incidence is an expression of the number of fissures with carious lesions. Since the cotton rat has 40 fissures per head the maximum caries incidence therefore would be 40.

<sup>2</sup> The extent of the caries of each fissure is assigned values of 1 + to 5 +; 1 + to 2 + = lesions involving only the enamel; 3 + = lesions involving the enamel and dentine; 4 + to 5 + = lesions resulting in fractures. The extent of carious lesions as used herein represents the sum of the individual fissure values.

growth averages were computed as growth of males by raising the figures for females by 20% at 6 weeks and 15% at 14 weeks (Anderson et al., '47). All animals grew well, although growth was usually somewhat better on the control ration than on the experimental diets.

The data on caries indices show that scores for all animals on the experimental diets were below those for animals fed the cariogenic control ration 802. The relative effect of the various experimental diets can be determined by comparing the differences between control and experimental averages in each case.

The caries scores of the rats fed dry milk rations were higher than the zero scores previously found on liquid whole milk. The average caries scores on dry whole milk were somewhat below those observed on dry skim milk plus butter. Thus, animals on dry whole milk had an average incidence of 8 and an average extent of 13 + as against the animals on control ration 802 which had an average incidence of 25 and 59 +, while animals on dry skim milk plus butter showed an average score of 14 and 30 + as against the controls on diet 802 with an average caries index of 28 and 70 +. It can also be seen that when these dry milk rations were reconstituted and fed in liquid form scores were reduced to nearly zero in both cases. Likewise, when the control ration 802 was mixed with water and fed in a liquid form, caries scores were found to be less than those of animals fed the same ration in a dry state, the averages being 9 and 14 + for experimental animals and 24 and 60 + for the controls on diet 802.

#### DISCUSSION

It appears from these data that fluidity of the ration is a very important factor in dental decay in the cotton rat. In comparison with the zero caries scores found with liquid whole milk, the caries scores on the dry milk rations fed in these experiments are high, but the incidence and extent of lesions were again reduced when these rations were reconstituted and fed in liquid form. Similarly, the caries scores of

animals receiving the ration 802 in a liquid form are considerably lower than those of their controls fed the same ration in a dry state.

Earlier experiments in this laboratory, using a synthetic dry ration approximating milk solids in composition, indicated that this ration produced a low incidence and extent of caries, although the caries scores were higher than the zero scores found on liquid whole milk (Schweigert et al., '46b). The caries indices found here on dry whole milk are above those observed on the previous dry milk solids ration, and the average for the animals fed dry skim milk plus butter is still higher. It is difficult as yet to explain these differences. It is possible that dissimilarities in composition existed between the dry milks used here and the synthetic milk solids ration fed in earlier experiments. Also, the percentage of fat in the skim milk plus butter ration is slightly below that usually found in milk solids. These differences may account for some changes in caries scores, since the levels of carbohydrate, fat, and protein in the diet have been found to affect caries production (Schweigert et al., '46a, '46b).

Differences in physical state other than fluidity may also have been influential. The drying process may alter certain milk constituents and thus make the effect of dried milk different from that of synthetic milk solids. Similarly, the condition of the fat may have been important in the difference between dry whole milk and dry skim milk plus butter. Free fat or oil in the diet has been postulated by Rosebury and Karshan ('39) and by McCollum et al. ('39) to reduce dental caries by forming a mechanical coating over the tooth or food particle, thus protecting against bacterial action. It is possible that butter added to the dry ration and mixed by hand may not coat the food particle as effectively as butterfat dried as part of the whole milk.

Fat may also enter into the effect of fluidity, although the condition of the fat in the reconstituted rations is unknown. Probably more important, however, is the possibility that these fluid rations, by remaining in the mouth for a compara-

tively short time, afford a less favorable medium for fermentation by oral bacteria. The differences in growth rate indicate that a lower caloric intake may also have had some effect.

The averages on the reconstituted milk rations are not zero as they have been found to be on liquid whole milk, but the differences may be within the limit of error of our procedure. The method of reconstitution was not entirely satisfactory and such small differences might also be accounted for by variations in physical state between these rations and liquid whole milk.

This, of course, does not preclude the possibility that other factors may also play a role in the protective action of milk. It is probable that the higher levels of fat and protein may exert some protection, and the presence of lactose as carbohydrate may enter into the picture, since it appears that this sugar is somewhat less cariogenic than the more fermentable ones. In addition, the effects of minerals and vitamins have been only partially investigated.

#### SUMMARY

Data are presented on the caries indices of cotton rats fed various dry and liquid rations.

Animals fed dry whole milk and dry skim milk plus butter were found to have an incidence and extent of carious lesions higher than the zero scores previously observed in animals fed only liquid whole milk. Caries scores were again reduced to nearly zero, however, when these rations were reconstituted with water and fed in liquid form.

In these experiments the caries indices of animals on all the diets were below those of control animals receiving the dry ration known to be highly cariogenic. Scores were also lower than those of controls when animals were fed the control ration in a liquid form. These data indicate that fluidity of the ration is an important factor in producing dental caries in the cotton rat.

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# THE NUTRITIVE QUALITY AND THE TRYPSIN INHIBITOR CONTENT OF SOYBEAN FLOUR HEATED AT VARIOUS TEMPERATURES<sup>1</sup>

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TWO FIGURES

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In their fundamental studies of the nutritive value of proteins, Osborne and Mendel ('12) found that glycinin, a protein isolated from soybeans, was of good nutritive value for rats, in comparison with a number of other proteins. The isolated protein was prepared by the method of Osborne and Campbell (1898), without the use of heat at any stage. Later, adopting the same methods that they had followed previously, and using soybeans rather than the isolated protein, Osborne and Mendel ('17) found that the protein of raw soybeans was of poor quality, and that heating the soybeans in the presence of water improved their nutritive value considerably. The earlier of these observations seems to have escaped general attention.

Mitchell and Smuts ('32), Shrewsbury and Bratzler ('33), and others found that the protein quality of raw soybeans

<sup>1</sup> Journal paper no. 321 of the Purdue University Agricultural Experiment Station.

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was improved by supplementation with cystine, and Hayward and Hafner ('41) found that methionine was even more effective in this respect. Johnson, Parsons and Steenbock ('39) showed that, although the fractions of N and of S absorbed by rats from heated soybeans were only slightly larger than the fraction of each element absorbed from raw soybeans, the retention of each element was greater with the heated soybean meal. They suggested that there is present in raw soybeans "a N and S containing complex which is absorbable but not usable for tissue building purposes." The work of Evans and McGinnis ('46) and of Evans, McGinnis and St. John ('47), however, indicated that chicks fed raw soybean meal excreted a large proportion of the absorbed S in the feces rather than in the urine. They found that the loss of cystine in the feces was especially large.

The presence of a substance in soybeans that inhibited the proteolytic action of pancreatin was demonstrated by Ham and Sandstedt ('44) and by Bowman ('44). Adding a concentrate of the inhibitor to an otherwise adequate diet decreased the growth rate of chicks, as shown by Ham, Sandstedt and Mussehl ('45). Klose, Hill and Fevold ('46) showed that a similar concentrate would decrease the growth rate of rats, although this concentrate was not specifically identified as an inhibitor of a proteolytic enzyme. A crystalline protein with strong trypsin-inhibiting activity was isolated from soybeans by Kunitz ('45, '46), who later ('47) characterized its properties, showing its strong affinity for trypsin and its much weaker affinity for chymotrypsin. Bowman ('46) presented evidence that more than 1 trypsin inhibitor is present in soybeans.

It appeared probable that the trypsin inhibitor caused the difference in nutritive value between raw and heated soybeans. The results of the present investigation confirmed this assumption. Studies were also made of the period and the temperature of heating necessary for the experimental preparation of soybean flour of optimal protein quality.

## EXPERIMENTAL

A commercial solvent-extracted soybean flour<sup>3</sup> was used in this study. For heating the flour at temperatures below 100°C., a suspension of the flour in water was maintained thermostatically at the desired temperature. After the required period, the suspension was cooled rapidly, lyophilized, and the dry product ground to pass a 40-mesh screen. For heating at temperatures above 100°C. the autoclave was used, with the flour spread in a thin layer. After the heating period, the flour was air-dried and ground to pass a 40-mesh screen.

Soybean protein was prepared from the raw flour by the method of Smith and Circle ('39), using pH 6.7 for extraction, and pH 4.1 for precipitation, of the protein. A concentrate of the trypsin inhibitor was prepared by lyophilizing the supernatant solution remaining after the precipitation of the protein.

The nutritive value of the proteins was determined by the mouse method of Bosshardt, Ydse, Ayres and Barnes ('46). A well-heated soybean flour was used in preliminary experiments to determine the optimal dietary level for soybean protein. Since maximal utilization of N for growth was indicated at 12% protein ( $N \times 6.25$ ), this level was used for all diets in which the protein was derived mainly from soybeans. Diets containing casein provided 10% protein ( $N \times 6.25$ ). The results were calculated as grams of weight gained per gram of protein consumed. Analyses of the carcasses for N showed that the weight gains were not due to an excessive deposition of non-protein constituents in the tissues.

The percentage composition of the diets was as follows: cerelese 20, hydrogenated vegetable oil 25, salt mixture (Hubbell, Mendel and Wakeman, '37) 4, Cellu flour<sup>4</sup> 2, corn oil 2, liver extract<sup>5</sup> 1, choline chloride 0.2, a vitamin A, D, and E concentrate 0.1, a vitamin B mixture 0.0372, and 2-methyl-1,4-

<sup>3</sup> Nutrisoy XXX flour, generously provided by Dr. J. W. Hayward of the Archer-Daniels-Midland Company.

<sup>4</sup> Obtained from the Chicago Dietetic Supply House, Chicago, Illinois.

<sup>5</sup> Wilson's 1:20 liver extract.

naphthoquinone diacetate 0.001, together with the required amount of the protein source and enough white dextrin to make 100. The vitamin A, D, and E concentrate contained 41 gm of corn oil, 2 gm of  $\alpha$ -tocopherol, and 7 gm of a fish-liver oil concentrate containing 65,000 U.S.P. units of vitamin A and 13,000 U.S.P. units of vitamin D per gram. The vitamin B mixture was composed of 200 mg of thiamine hydrochloride, 400 mg of riboflavin, 200 mg of pyridoxine hydrochloride, 1,000 mg of niacinamide, 1,100 mg of calcium pantothenate, 5,400 mg of inositol, and 1,000 mg of p-aminobenzoic acid.

#### *Determination of trypsin inhibitor potency*

A method was developed for determining the trypsin inhibitor potency of raw or heated soybean flours. The method was the same in principle as Anson's ('38) method for trypsin determination. It differed from the method recently described by Borchers, Ackerson and Sandstedt ('47) for the determination of soybean trypsin inhibitor in the use of casein as the substrate instead of hemoglobin. Crude trypsin,<sup>6</sup> referred to hereafter as "pancreatin" to avoid confusion with pure trypsin, was used instead of the pure enzyme, because of the difficulty of obtaining the latter. In this method, 5 ml of 2.5% casein<sup>7</sup> solution and 1 ml of an inhibitor solution, or of water, were placed in each of a series of tubes. After warming the tubes to 35°C., 1 ml of a standard solution of pancreatin was added to each tube, and the incubation continued for exactly 20 minutes. To each tube 10 ml of 10% trichloroacetic acid was added, after which the tube was shaken and allowed to stand for 1 to 3 hours. The suspension was filtered through a dry paper, and a 10 ml aliquot taken for N determination by a micro-Kjeldahl method.

The pancreatin solution was standardized to release from 1.8 to 2 mg of N in the aliquot analyzed, in the absence of any

<sup>6</sup> Wilson's 1:300 trypsin.

<sup>7</sup> "Labco" casein.

inhibitor. The N in the aliquot was proportional to the amount of pancreatin when less than 2 mg of the enzyme was used. To obtain reproducible results, it was necessary to use a standardized enzyme solution, which was preserved by freezing in small portions. Each portion was thawed just before using.

Provided the amount of inhibitor present did not reduce the amount of N released by more than 35%, the decrease of the N released was proportional to the amount of inhibitor used. The amount of N released in each tube containing the inhibitor was deducted from the value obtained in the absence of any inhibitor. The difference was divided by the amount of inhibitor material added. An average of 8 or more determinations was used to obtain the inhibitor potency, expressed as milligram of N per milligram of test substance.

In assaying the heated soybean flours, thorough extraction of the inhibitor was necessary to obtain reproducible results. Satisfactory extraction was obtained by adjusting a 4% to 5% suspension of the flour to pH 10.5 for 30 minutes. Subsequent dilution to give the desired concentration of the inhibitor lowered the pH sufficiently so that no neutralization was necessary. These conditions caused no destruction of the inhibitor.

*The relationship between inhibitor potency and  
protein efficiency*

Separate portions of soybean flour were heated for 30 minutes at 60°, 65°, 75°, 85°, 95°, and 108°C. to yield products of decreasing inhibitor contents. The protein efficiency and the trypsin inhibitor potency of each heated portion were determined. The results (fig. 1) indicate that within the limits of accuracy of the methods used, the nutritive value of the protein increased in proportion to the destruction of the inhibitor. The results with soybean flour of higher inhibitor potency than the flour heated at 60°C. are not reported, because the

mice lost weight to a variable extent with such levels of inhibitor in their diets.

The isolation of soybean protein by the method described leaves a considerable proportion of the inhibitor in the product. The protein efficiency of this preparation was in approximate agreement with the value expected from the results with partially heated flours (fig. 1).

The inhibitor concentrate was added to a diet with autoclaved soybean flour, at a level permitting comparison with the previous results. The agreement with the expected value

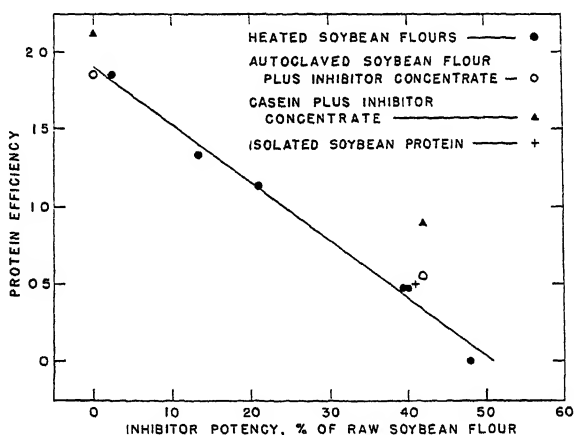


Fig. 1 The relationship between protein efficiency and trypsin inhibitor potency.

was also satisfactory (fig. 1). In this case, the inhibitor concentrate may have supplemented the autoclaved soybean flour to some extent, since the autoclaved concentrate, when fed with the autoclaved flour, gave a protein efficiency of 1.35 as compared with 1.65 for the autoclaved flour alone.

The effect of the inhibitor concentrate upon the nutritive value of casein was considerable, although proportionately less than upon that of soybean flour. In this instance, the inhibitor concentrate did not supplement the casein. These results also are given in figure 1.

*The effect of different periods and temperatures of heating upon the nutritive value of soybean flour*

Soybean flour was autoclaved at 108°C. (5 lbs.) and at 120°C. (15 lbs.) for different periods. The protein efficiencies of the products are shown by figure 2. It can be seen that, not only was the protein damaged by continued heating at 120°C. in the autoclave, but also that a soybean flour of optimal protein quality was not produced with any heating period at 120°C.

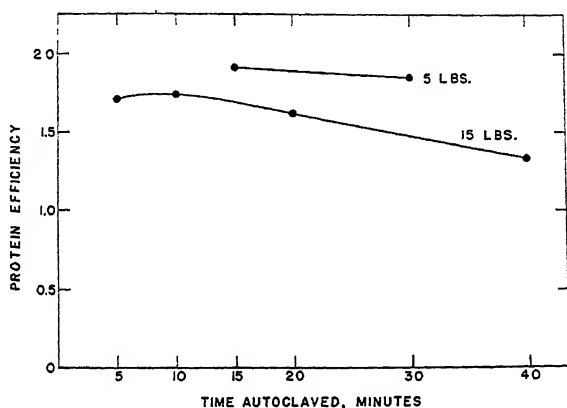


Fig. 2 The effect of different periods and temperatures of autoclaving upon the protein efficiency of soybean flour.

However, no significant loss of nutritive value resulted from extending the heating period from 15 minutes to 30 minutes at 108°C. Moreover, both results were essentially equivalent to the highest possible protein efficiency, as indicated by the extrapolation of the results for flours heated at lower temperatures.

#### DISCUSSION

Several of the observed relationships indicate that the trypsin inhibitor is the major cause of the poor utilization of the protein of raw or of partly heated soybeans. These relationships may be summarized as follows: (1) The protein efficiency of partially heated soybean flours increased in direct

proportion to the destruction of inhibitor potency, provided the temperature used was not over 108°C. (2) Adding the trypsin inhibitor concentrate to autoclaved soybean flour depressed the protein efficiency to the extent expected from the results with the partially heated soybean flours. (3) Isolating soybean protein by a process not involving heat improved the nutritive value to the extent expected from the proportion of inhibitor potency removed. (4) The highest protein efficiency attainable for the soybean flour used, as indicated by extrapolation to zero inhibitor potency, was the value reached under the heating conditions found to be optimal.

The evidence cited applies specifically to mice. While confirmation with other species would be desirable, the evidence available indicates that the effect of the inhibitor on mice differs only in magnitude from its effect on other species. However, it is clear that the inhibitor affects mice more strongly than it does rats or chicks. For example, Klose et al. ('46) obtained a protein efficiency of 0.7 for raw soybean flour fed to rats, compared with the present results showing a marked loss of weight by mice fed raw soybean flour. The reason for this difference is not known at present.

The harmful effect of high temperatures on soybean flour also may be greater for mice than for other species. Nevertheless, since the trypsin inhibitor is the cause of the poor utilization of raw soybean protein, the minimal heating conditions for destroying the inhibitor should yield a soybean flour of optimal quality for any species. Therefore, the determination of the trypsin inhibitor potency might be of practical value in controlling the heating processes used in the commercial production of soybean oil meal. Since the presence of a small amount of inhibitor potency would not have any significant effect, the optimal heating period for any particular process would be the time required to destroy all but a few per cent of the original inhibitor potency. However, the inhibitor determination cannot give any information regarding the deleterious effect of excessive heat. To measure this, other tests must be used.

The optimal temperature for heating soybeans, as indicated by the present investigation, is in agreement with the results reported by others. Parsons ('43), using rats, obtained the best product by autoclaving at 110°C. for 30 minutes, and Evans and McGinnis ('46), with chicks, obtained the best products by autoclaving 30 minutes at 100°C. or 110°C. Clandinin, Cravens, Elvehjem and Halpin ('46), however, reported that autoclaving for 4 minutes at 140°C. gave a good quality product. While differences in the heating techniques used experimentally would affect the products, it is believed that the evidence presented shows clearly that autoclaving at 108°C. is superior to 120°C., and that equally good results may be obtained at 108°C., whether the heating period is 15 or 30 minutes.

While the evidence presented supports the view that the trypsin inhibitor is the chief cause of the poor utilization of raw soybean protein, other data are required to explain the mechanism of its action. Since its action is manifested not only with soybean protein, but also with casein, and since Ham et al. ('45) showed that this inhibitor decreased the utilization of a practical ration by chicks, it appears that its action is not due to a specific configuration of the major proteins of the diet.

#### SUMMARY

1. A method was developed for determining the trypsin inhibitor potency of raw or heated soybean flours.

2. The protein efficiency of soybean flour increased in direct proportion to the destruction of its trypsin inhibitor potency by heat.

3. The protein efficiencies of autoclaved soybean flour to which the inhibitor had been added, and of isolated soybean protein partially freed of the inhibitor, agreed well with the values predicted from the results with heated flours. It was concluded that the presence of the trypsin inhibitor was the chief cause of the poor utilization of the protein or of inadequately heated soybean flours.



4. Autoclaving at 108°C. for 15 to 30 minutes produced a soybean flour of optimal protein efficiency for mice, while autoclaving at 120°C. for 5 or 10 minutes resulted in a slightly poorer product. Autoclaving for periods of over 10 minutes at 120°C. caused a progressive decrease in the protein efficiency of the flour.

5. On the basis of this evidence, it is suggested that the determination of the inhibitor potency of soybean products may be used as a practical index of the effectiveness of the heating to increase the protein quality, providing that the heating is not continued beyond the time required for the total destruction of the inhibitor.

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# THE RELATION OF THIAMINE CLEARANCE TO BLOOD LEVELS IN MAN

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ONE FIGURE

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Since thiamine blood levels (Goodhart and Sinclair, '40) and urinary clearance values (Melnick and Field, '42) have been proposed as indications of thiamine status, it was thought desirable to study by both methods a number of subjects of varying nutritional sufficiency to determine the relationship between the results.

## METHOD

### *Selection of subjects*

The 23 subjects reported in this study were patients in the wards of a general hospital. Since it was impossible to obtain a reliable estimate of recent thiamine intake in each case, selection was directed solely toward securing a group with as wide a range of nutritional status as possible. Patients receiving sulfonamide drugs were excluded because of the possible adverse effect on the microbiological assay for thiamine used. The 2 estimations were repeated on 1 patient only. No cases of uncomplicated vitamin deficiency were available for study. Three of the subjects had been receiving thiamine therapy in the form of multivitamin tablets for varying periods before the estimations were made.

*Test dose procedure and collection of specimens*

After the subjects had emptied their bladders (8:30 A.M.), fasting specimens of blood were collected in heparin by venipuncture. Immediately after withdrawal of the blood, an intramuscular injection (gluteal) of 350  $\mu$ g of thiamine chloride hydrochloride in saline per square meter of body surface was given.

Urine was collected covering a period of 4 hours after the injection, with the subjects in a fasting state. The brown collection bottles contained 10 ml of 5N HCl and a few drops of toluene. Since previous work in this laboratory had shown that a false low excretion may result from incomplete passage of a small specimen of very high thiamine concentration, water was given by mouth in an effort to obtain urine specimens of adequate volume.

*Preparation of specimens and analysis*

Blood specimens were centrifuged at 2500 R.P.M. for 20 minutes and the hematocrit values read. After the plasma had been pipetted off, the blood cells were resuspended in saline to the original volume of blood centrifuged. A 4-ml volume of the saline suspension of cells was pipetted into 80 ml of 0.5% acetate buffer, pH 4.5, containing 50 mg each of papain and takadiastase. This preparation was allowed to digest at room temperature for 24 hours and prepared for analysis as described by Sarett and Cheldelin ('44).

Upon completion of the collection period, the urine specimens were steamed to remove the toluene. An aliquot, depending on the volume and estimated content of thiamine, was brought to pH 6.5 and diluted to volume for analysis. Undiluted specimens were kept at  $-15^{\circ}\text{C}$ . for additional analyses if necessary.

Both urine and blood specimens were analyzed for thiamine by the *Lactobacillus fermentum* method (Sarett and Cheldelin, '44; Cheldelin et al., '46). The pyrimidine and thiazole derivatives of the thiamine molecule had little if any activity under

the conditions of the collection and analytical method used. The sensitivity of the method enabled adequate dilution of urine specimens to prevent "drift" occasionally seen in this type of assay. Each specimen was set up for assay at 6 to 8 dilutions, together with recoveries of 0.005  $\mu\text{g}$  added thiamine at 3 levels. All assays were checked for "validity" by the method of Wood ('45). A deviation of  $\pm 10\%$  from the mean result was considered the maximum permissible in any of the levels run, although usually the deviations fell within a  $\pm 5\%$  range.

The "P" value of Fisher ('32) was applied to correlation coefficients as a test of significance because of its suitability for small samples. A "P" value of 0.01 or less was required before a correlation was considered significant.

All references in this report to micrograms ( $\mu\text{g}$ ) of thiamine should be read as "micrograms of thiamine chloride hydrochloride."

## RESULTS

Physical measurements and laboratory findings are presented in order of thiamine excretion in table 1. Total thiamine excreted in 4 hours following administration of the test dose is plotted against blood cellular thiamine in figure 1.

Since Melnick and Field ('42) found clearance values below 50  $\mu\text{g}$  invariably associated with thiamine deficiency, subjects excreting less than this amount were reviewed to test the possibility of dividing the series into 2 distinct groups. Although no clinical criteria for such a division could be arrived at, the thiamine clearance values of severely ill patients (subjects 16, 17, 18, 20, 21, 22 and 23) were generally low regardless of the etiology of their condition, while convalescent subjects and those in hospital for investigation of relatively mild conditions (subjects 1, 2, 5, 6, 7b and 9) usually excreted larger amounts of the test dose.

Rapidly changing food intakes made it difficult to assess the influence of this factor on the clearance results except in known cases of thiamine therapy. High levels of intake (10

to 20 mg thiamine per day) for a few days did not produce as high a clearance as might be expected (subjects 8 and 12), while an intake of 3 mg per day for a month was associated with an excretion in the "normal" range (subject 3).

TABLE 1  
*Data of present study.*

SUB- JECT NO.	AGE	WEIGHT	URINE VOLUME	THIAMINE EXCRETED	THIAMINE INJECTED	HEMATO- CRIT	BLOOD CELLULAR THIAMINE	
							per 100 ml blood	per 100 ml cells
		<i>lbs.</i>	<i>ml</i>	<i>μg</i>	<i>μg</i>		<i>μg</i>	<i>μg</i>
1	35	134	330	139	580	35	3.8	10.8
2	34	138	285	180	600	49	12.8	26.0
3	65	142	160	115	580	37	12.3	33.2
4	53	160	190	111	610	45	9.0	20.0
5	77	160	995	111	640	48	6.8	14.2
6	18	170	390	87	690	49	7.0	14.2
7	34	112	850	75	540	41	5.3	13.0
7b	34	112	830	116	540	46	8.5	18.4
8	40	142	330	74	640	47	5.3	11.2
9	42	175	110	66	630	42	6.3	15.0
10	46	110	225	60	520	30	5.0	16.6
11	40	200	1230	57	680	45	7.5	16.6
12	13	96	70	53	490	50	8.8	17.6
13	75	150	720	48	580	54	6.5	12.0
14	66	143	85	44	570	45	7.1	15.8
15	60	128	135	37	570	46	8.5	18.4
16	74	106	170	34	530	45	7.3	16.2
17	79	92	870	30	480	34	4.5	13.2
18	71	98	180	25	520	46	10.0	21.8
19	37	97	283	23	500	42	9.5	22.6
20	55	131	65	20	600	35	7.3	20.8
21	50	107	145	13	750	42	9.8	23.4
22	75	122	55	9	540	41	11.3	27.6
23	64	152	220	6	630	25	4.5	18.0

The wide range of 10.8 to 27.6  $\mu\text{g}$  thiamine per 100 ml of blood cells corresponded approximately to that reported for blood cocarboxylase in normal men by Goodhart and Sinclair ('39). High blood levels were found in subjects that excreted either large or small amounts of thiamine, with low levels

occurring mostly in those having intermediate clearance values of 60 to 100  $\mu\text{g}$ . The highest and lowest blood levels were found in the group with clearance values in the "normal" range of 90  $\mu\text{g}$  and over.

Consideration of the results suggested a division of the subjects into 2 groups (fig. 1): those who excreted less than 90  $\mu\text{g}$  thiamine (group A) and those who excreted over this

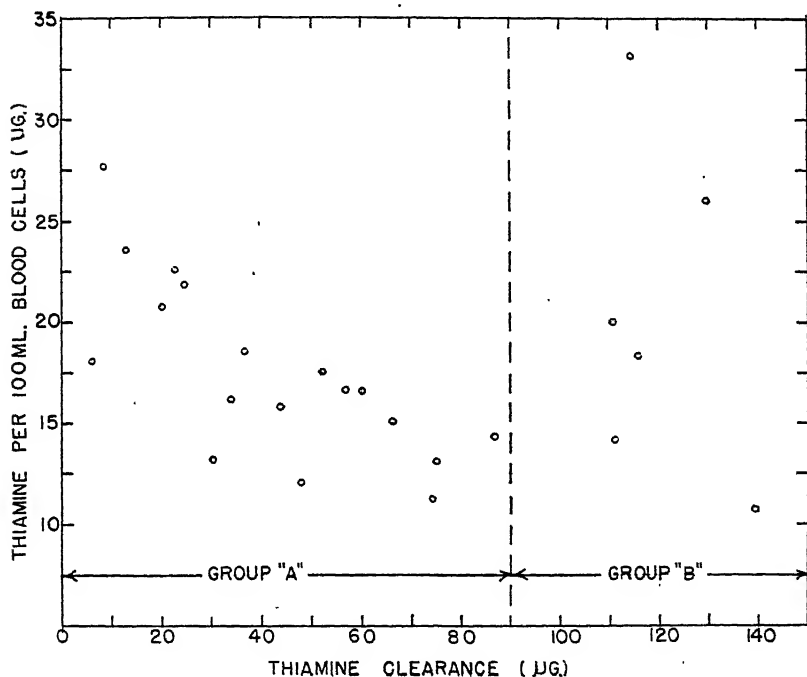


Fig. 1 Blood cellular thiamine of subjects plotted against thiamine clearance. Groups A and B are explained in the text.

amount (group B). While group B was too small for statistical analysis, it would seem that no relation existed between blood and excretion values. However, a statistically significant correlation coefficient of  $-0.74$  was found for these values in group A. The corresponding correlation coefficient of  $-0.08$  for groups A and B taken together was not significant. In addition, a significant correlation of  $-0.62$



between age and thiamine clearance was obtained in group A, with only a moderately significant value ( $-0.37$ ,  $P=0.05$ ) in groups A and B taken together. Further attempts to correlate thiamine output and blood levels with other variables such as weight and urine volume were unsuccessful.

It will be noted that blood levels are expressed throughout as micrograms of thiamine per 100 ml of blood cells. This is purely an arbitrary measure dependent upon the finding that these derived results showed a slightly better correlation with clearance than the corresponding values of micrograms of cellular thiamine per 100 ml of blood.

#### DISCUSSION

Although our series could not be divided into 2 groups corresponding to the "normal" and "deficient" clearance ranges of Melnick and Field ('42), there was a definite association of low clearance values with poor general condition. The correlation of lowered thiamine excretion with increased age was considered to be a reflection of the general debility of our elderly subjects.

The simplest explanation for the negative correlation between the blood and clearance values in group A would seem to be on the basis of renal clearance. If such a mechanism is applicable to our series, defective renal clearance in the debilitated, elderly subjects would result in a decreased urinary excretion with a rise in plasma thiamine leading to a rise in blood cellular thiamine. Before such a view can be accepted, it would be necessary to show that the cellular levels are dependent, at least in part, upon the plasma levels. This problem is being investigated.

Regardless of the physiological interpretation applied to these results, they do suggest that some caution should be exercised in using these methods for the classification of subjects into "normal" and "deficient" groups.

#### SUMMARY

1. Simultaneous blood thiamine and thiamine clearance estimations were made on 23 hospital patients.

2. Low clearance values were found in the more severely debilitated subjects regardless of the etiology of their condition, while larger amounts of thiamine were excreted by convalescent subjects and those under investigation for minor ailments.

3. In the group excreting less than 90  $\mu\text{g}$  thiamine, higher blood levels were found in the more severely ill patients.

4. A significant negative correlation was found between blood cellular thiamine and thiamine clearance values below 90  $\mu\text{g}$ . A similar correlation of age with clearance values was considered a reflection of the general debility of the elderly subjects of the series.

#### ACKNOWLEDGMENTS

We wish to thank Dr. J. Doupe, Director of the Department of Medical Research, for numerous suggestions and help in providing laboratory and clinical facilities.

Merck and Company kindly donated cocarboxylase for standardization of the assay.

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## ERRATUM

WARKANY, JOSEF, AND CAROLYN B. ROTH. Congenital malformations induced in rats by maternal vitamin A deficiency.

Journal of Nutrition, vol. 35, no. 1, January, 1948.

Page 3: Line 17 and following should read: "yeast<sup>3</sup> 10; sodium chloride of C.P. grade, 1. Sixty I.U. of vitamin D and measured amounts of carotene were given every tenth day. According to the carotene supplements added," etc.

Page 4: In lines 6 to 13 (from top) all values expressed in micrograms ( $\mu\text{g}$ ) should be changed to milligrams (mg).



# THE DETERMINATION OF MINIMUM VITAMIN REQUIREMENTS FOR GROWTH<sup>1</sup>

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## THREE FIGURES

(Received for publication November 3, 1947)

The minimum requirement for growth of any nutrient may be defined as the lowest level of that nutrient which allows the maximum growth inherently possible. Such a definition tacitly assumes that maximum growth is optimum growth. This is obviously true if one is concerned with meat production, provided the growth is obtained economically, but is much less obviously so when intelligence, longevity, milk production, etc., are the prime considerations. However, the definition appears to be generally accepted as applying to human beings, since recommended allowances have always been set at levels thought to be considerably above minimum requirements. It is also generally accepted that *minimum requirements should adequately cover the needs of all individuals*.<sup>2</sup> If this view is adopted, then the average requirement loses importance if there is a large variation in the data from which it was derived. That is, if there is a considerable

<sup>1</sup>Supported by grants-in-aid from the John and Mary R. Markle Foundation, New York, New York; Swift and Company, Inc., Chicago, Illinois; Milbank Memorial Fund, New York, New York; and the Nutrition Foundation, Inc., New York, New York.

<sup>2</sup>This statement should perhaps read "99% or 99.9% of all individuals." If the vitamin requirements of individuals fit a normal distribution, those of a few would be infinitely high.

range in individual needs, a diet which supplies only enough to meet average needs will be too low for a considerable portion of the population. It will also, of course, contain more than enough for a similarly large fraction, but this would appear to be of relatively little importance unless the cost of the nutrient must be carefully considered along with availability.

It would thus appear that the chief danger in estimating requirements is that they will be underestimated since little attention has been paid to differences in the needs of individuals within the population studied. If the range of requirements could be estimated, the recommended allowance could be made with some assurance rather than being arrived at simply by doubling or tripling the minimum needs. Although this has not been accomplished, it does appear to the author that the usual methods of evaluating the data from experiments designed to determine minimum requirements for growth tend to underestimate the needs, and that less critical methods may be justified. In this paper, data collected on the riboflavin requirements of ducklings are used as examples for discussion since they are more or less typical of material which must be evaluated.

#### EXPERIMENTAL

The experimental details are presented in the paper by Hegsted and Perry ('48). Pekin ducklings 3 or 4 days old were divided into groups of 4 or 5 ducks each and fed purified rations containing different levels of riboflavin ranging from 100 to 500  $\mu\text{g}$  per 100 gm of diet.<sup>3</sup> After 2 weeks the gain per day during the last 10 days on experiment was calculated. The effect of body stores on growth should have been minimized by using only the latter part of the study in the calculation.

<sup>3</sup> The crystalline vitamins other than folic acid used in these studies were kindly supplied by Merck and Company, Rahway, New Jersey; the glucose and corn oil by the Corn Industries Research Foundation, New York, New York; and the folic acid by the Lederle Laboratories, Pearl River, New York.

The problem in the analysis of the data is to determine at what level of riboflavin fed maximum growth is reached. It is also of interest to determine, if possible, whether the response to riboflavin is linear over the entire range of riboflavin supplementation to the point where maximum growth is obtained. While it is unlikely that this latter proposition is true, if it were approximately true the analysis would be greatly simplified. It seemed likely that the response might be more nearly linear when the logarithm of the dose was used rather than the dose itself. Various methods of examining the data which have previously been used were therefore compared, namely (a) plotting the mean gains of the various groups against the riboflavin content, (b) plotting individual gains against riboflavin content, and (c) plotting individual gains against the log of the riboflavin content. In the latter 2 instances the best regression lines were calculated by the method of least squares through the values for the first 3 groups (100 to 300  $\mu\text{g}$  riboflavin/100 gm diet), the first 4 groups (100 to 400  $\mu\text{g}$  riboflavin), and the 5 groups (100 to 500  $\mu\text{g}$  riboflavin).

## RESULTS

### *Mean gain vs. riboflavin level*

In figure 1 the mean gain of each of the groups is plotted against the riboflavin content of the diet and a smooth curve drawn through the points by inspection. This is the usual treatment of such data and casual inspection suggests a requirement of about 350  $\mu\text{g}$  of riboflavin per 100 gm of diet. Application of the "t" test does not reveal any significant difference in the gain at the 300 and 400  $\mu\text{g}$  levels of riboflavin in any of the experiments or in the combined data.<sup>4</sup>

### *Individual gain vs. riboflavin level*

When the individual gains are plotted in a similar manner (fig. 2), the requirement for maximum growth is far from

<sup>4</sup>All of the data have been tested at 0.05 probability level for significance. Since the paper is a general discussion of the problems involved, the statistical tables have not been presented.



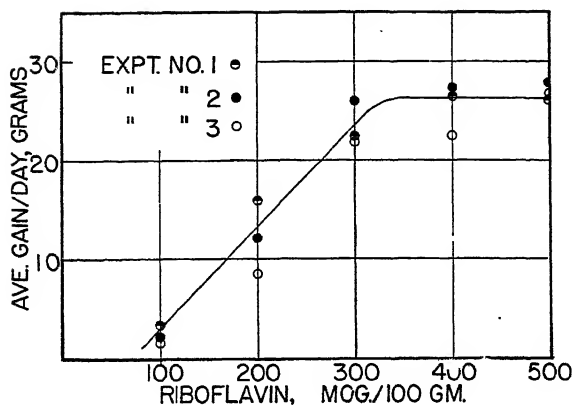


Fig. 1 The average gain per day of the groups of ducklings receiving various levels of riboflavin.

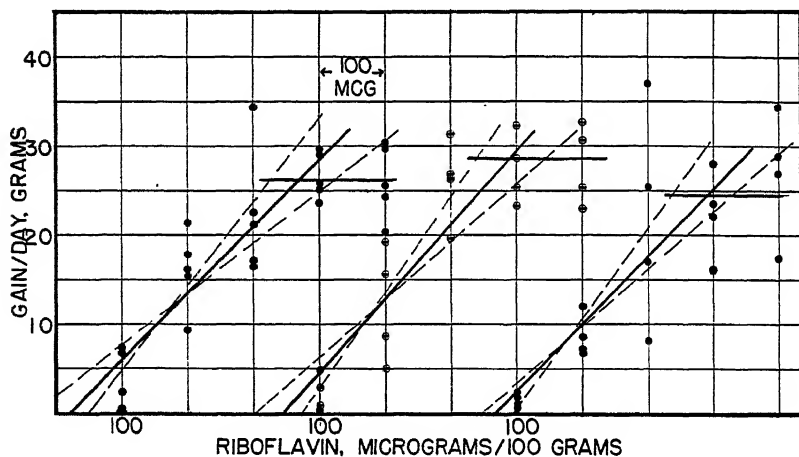


Fig. 2 Scatter diagram showing the gain per day of each duckling versus the riboflavin content of the diet. The solid line is the regression line through the points for the first 4 levels of riboflavin. The solid horizontal bar is drawn through the mean weight of the last 2 groups. The dotted lines are the regression lines for the first 3 groups and all 5 groups.

clear. As judged by the correlation coefficient (table 1) the correlation between gain and riboflavin level is as good over the total range tested, from 100 to 500  $\mu\text{g}$  riboflavin, as it is for the first 3 levels from 100 to 300  $\mu\text{g}$ . The various groups of data were also examined for departure from linearity by the covariance method described by Fisher ('46). As might be expected, none of the data depart significantly from linearity

TABLE 1

*Riboflavin requirements as estimated from different regression lines and 2 methods of plotting the data.*

EXPERIMENT NUMBER	RANGE OF RIBOFLAVIN IN REGRESSION LINE IN $\mu\text{g}/100 \text{ GM}$	UNITS ON ABSCISSA					
		Riboflavin $\mu\text{g}/100 \text{ gm}$			Log riboflavin $\mu\text{g}/100 \text{ gm}$		
		$r^1$	Riboflavin requirement		$r^1$	Riboflavin requirement	
			Growth <sup>2</sup>	Maintenance <sup>3</sup>		Growth <sup>2</sup>	Maintenance <sup>3</sup>
1	100-300	0.837	327	47.6	0.855	372	81.3
	100-400	0.868	373	24.0	0.906	380	81.2
	100-500	0.826	434	—38.0	0.853	444	67.3
2	100-300	0.826	341	74.6	0.889	377	95.1
	100-400	0.845	390	48.6	0.901	405	92.5
	100-500	0.824	448	20.0	0.902	450	87.0
3	100-300	0.789	338	94.3	0.759	403	98.6
	100-400	0.797	396	69.7	0.804	434	96.4
	100-500	0.810	431	46.7	0.829	437	96.0

<sup>1</sup> Correlation coefficient.

<sup>2</sup> Riboflavin content at intersection of the regression line with the horizontal line through the mean gain at 400 and 500  $\mu\text{g}$  per 100 gm.

<sup>3</sup> Riboflavin content at intersection of the regression line with the horizontal line through zero gain.

although there is a tendency for greater departure as the range of riboflavin is increased.

If one assumes that the gain over any of the riboflavin ranges tested is linear with respect to riboflavin concentration, then the requirement for maximum growth would be that value of riboflavin at which the regression line reaches a value taken as maximum gain. In these studies we have taken the mean gain of the last 2 groups as maximum gain.

In figure 2 a solid line is drawn horizontally through this value, and the riboflavin value at which the regression line meets this line may be read off of the graph. These values are shown in table 1 as the growth requirement determined by each regression line.

*Individual gain vs. log riboflavin level*

In figure 3 the data have been plotted as in figure 2 except that the log of the riboflavin content of the diet has been used as the unit of the abscissa. The 3 regression lines

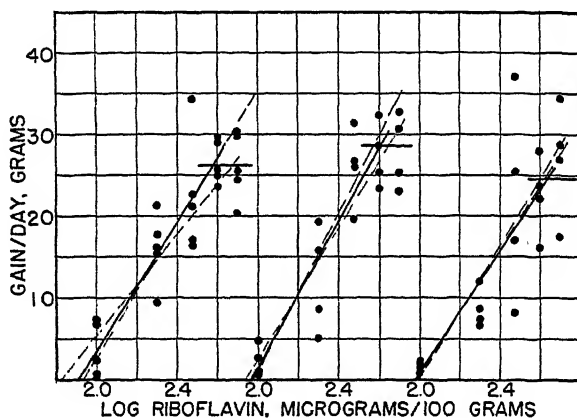


Fig. 3 Scatter diagram showing the gain per day for each duckling versus the logarithm of the riboflavin content. See the legend of figure 2 for explanation of the regression lines.

for each experiment were calculated as before and the requirement for maximum growth read off again as the riboflavin value at which the regression line reaches maximum gain. These values are shown in table 1. It may be seen that the requirement thus determined is less influenced by the choice of the data through which the regression line is calculated than is the case in figure 2. Also it may be noted that in general the use of log riboflavin has increased the correlation coefficient but not to a significant degree.

*Maintenance requirement*

If the point at which the regression line meets a horizontal line through maximum gain be taken as the requirement for growth, then in an analogous manner the riboflavin value giving zero gain should be the maintenance requirement. These values have also been reported in table 1 as determined in figures 2 and 3 for the various regression lines. It may be seen that this value, whatever its significance, would appear to be fairly reliable when calculated from a log dose-response curve, but of little value as determined from the dose-response curve. If it should be shown that the maintenance

TABLE 2

*Statistics calculated from the combined data.*

STATISTIC	MICROGRAMS OF RIBOFLAVIN PER 100 GM DIET				
	100	200	300	400	500
Mean gain, gm	2.5	12.6	23.4	25.6	26.9
Standard deviation	2.46	5.35	8.02	2.43	4.91
Standard error of mean	0.71	1.54	2.32	0.70	1.42
Coefficient of variation	98.4	42.5	34.4	9.5	18.2

requirement is the same for animals of different weights and ages, this value might be useful as a means of determining adult requirements.

*Variability within groups*

The second premise made in the introduction was that "minimum requirements should be set sufficiently high to cover the needs of all individuals."<sup>5</sup> It is instructive here to examine the extreme limit of the gains in each group. In each experiment the group at the 300  $\mu$ g level of riboflavin has a lower limit than the groups at 400 and 500  $\mu$ g and the range of values is greater. This is demonstrated more clearly in table 2 in which several statistics have been calculated for the combined data from the 3 experiments. Of chief interest

<sup>5</sup> See footnote 2, p. 399.

is the coefficient of variation which tends to decrease with increasing levels of riboflavin. This suggests that at low levels of riboflavin supply one is dealing with more than 1 variable. These may be (a) inherent ability to gain and (b) response to riboflavin.<sup>6</sup> When sufficient riboflavin is supplied to meet the needs of all of the individuals, the second variable is largely removed and the groups become less heterogeneous.<sup>7</sup> It is not unlikely therefore that the 300  $\mu$ g level is adequate for a considerable proportion of the individuals, but those for which it is inadequate deserve considerable attention.

#### DISCUSSION

The determination of growth requirements is inherently more difficult than the usual vitamin assay and subject to much greater error. In vitamin assays the work can be done in that range of vitamin supplementation where the response is essentially linear to the dose or the log dose, and by choosing standards and unknowns in such amounts that an equal response is obtained, errors due to inaccuracies in the slope of the regression line may be largely eliminated. In studying requirements, however, one is interested in the extreme portions of the dose-response curve where response may not be linear with respect to dosage, and where errors in the slope of the regression line produce great errors in the value to be determined.

Previous work covering the assay of many vitamins has shown that the log dose-response curve is more nearly linear than the dose-response curve (Irwin, '37; Coward, '38). For the data considered in this paper, this appears to be highly probable although it is not possible to prove statistically. It would appear that the more general use of log dose-response

<sup>6</sup> It may be equally valid to explain the variation in terms of differences in food intake, and thus differences in riboflavin intake.

<sup>7</sup> It may be noted that the variance of the groups is not the same. According to Fischer ('46) this does not invalidate the common "t" test since one is interested only in knowing the probability that the 2 groups are from the same population. A significant value for "t" may be obtained either when the means are different or when the variances are different.

curves would have the following advantages in the determination of requirements: (a) The unreliability of the value obtained is emphasized. For example, on a log scale the difference between 300 and 400  $\mu\text{g}$  of riboflavin is relatively small while on an ordinary scale this represents a large portion of the range being considered. (b) The value obtained for the growth requirement is less dependent upon the judgment of the investigator in selecting the region over which it is valid to calculate the regression line. (c) The maintenance requirement thus determined appears to be a relatively reliable figure.

On the other hand, the usual test is to compare statistically the gains at 2 levels of vitamin supplementation to determine whether 1 level gives a significantly better response, such as the 300 and 400  $\mu\text{g}$  riboflavin level in the present data. This is at best a highly uneconomical procedure for, in effect, one discards the major portion of his data in making the test, and uses the data from only 2 small groups at exactly the point where large numbers are required to prove small differences significant. The statistician will correctly suggest that the experiment should be repeated with a new design so that large numbers fall within these groups. This, however, proves to be highly impractical in most instances. If one combines all of the data available at the 300 and 400  $\mu\text{g}$  levels of riboflavin, one may calculate that at least 70 birds would be needed to show the mean gains to be significantly different. Following this, even larger numbers would be required to test the differences between the 400 and 500  $\mu\text{g}$  levels. Few laboratories could undertake such a problem.

In comparing 2 groups of data, one is ordinarily testing the hypothesis that the 2 groups are the same, i.e., from the same population. Failure to find the differences to be significant may mean that the groups are the same or that the data are insufficient to prove the significance of the difference. It does not prove that the groups are the same. Therefore one must consider the likelihood of making a false decision, and in the small groups usually available in nutrition work,

it is apparent that a false decision will often be reached. One is more likely to conclude that 2 groups are not different when they actually are, than the reverse. Since this is also the more serious error, if one wishes to have requirements high enough to cover the whole population, then he must not rely completely upon the statistical test (see Eisenhart and Wilson, '43, for a clear example of this point).

While every effort should be made to increase the extent of the data and to apply appropriate statistical examination, the judgment of the investigator must not be completely discarded. The present data on the riboflavin requirement of the duckling are inadequate to prove statistically that more than 300  $\mu$ g of riboflavin per 100 gm of ration are necessary, but other considerations lead us to conclude that probably the requirement is not less than 400  $\mu$ g and that 500  $\mu$ g per 100 gm may be a safer level.

#### CONCLUSION

The usual method used in the determination of growth requirements is to feed various levels of the nutrient and then to test statistically the difference in response between the various levels to determine at which level one ceases to obtain a significant increase in response. Almost invariably small groups of animals must be used. Thus, most of the data are discarded in making the critical test, at exactly the point at which large groups are required to show the small differences to be significant, and the most likely error is that one will conclude that the groups are not different when in fact they may be. This type of error will invariably lead to a low estimate of requirement. A low estimate is considered a more serious error than the less likely over-estimation since one is interested in setting requirements high enough to cover adequately all of the individuals.

It is suggested that by plotting a log dose-response curve and determining the point at which the regression line, drawn through the region of approximately linear response, reaches maximum growth one arrives at a fairer estimate of the true

requirement. The maintenance requirement, that amount of nutrient required to give just zero gain, may also be determined with considerable accuracy from the same regression line.

#### ACKNOWLEDGMENTS

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# NUTRITIONAL STUDIES WITH THE DUCK

## V. RIBOFLAVIN AND PANTOTHENIC ACID REQUIREMENTS <sup>1</sup>

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### ONE FIGURE

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In previous publications in this series we have reported studies on the pyridoxine (Hegsted and Rao, '45) and niacin (Hegsted, '46) requirements of ducklings. The present paper presents the results of investigations on the riboflavin and pantothenic acid requirements of this species.

### EXPERIMENTAL

The rations <sup>2</sup> used in this study were of the purified type and had the compositions shown in table 1. Either riboflavin or calcium pantothenate was omitted to produce the appropriate vitamin low diet. Day-old Pekin ducklings were received from the hatchery and fed a commercial diet for 3 or 4 days. They were then divided into groups of 4 or 5 ducklings each and given the experimental diets to which had been added various levels of the vitamin under study. Each bird was weighed every other day. The experiments were terminated after 2 weeks and the gain per day during the last 10 days was

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<sup>2</sup> Folic acid was supplied by the Lederle Laboratories, Pearl River; and the other crystalline vitamins by Merck and Co., Rahway. Glucose and corn oil were supplied by the Corn Industries Research Foundations, New York.

calculated for each bird. By using only the data obtained during these last 10 days of the experiment, the influence of body stores on growth should have been minimized.

## RESULTS

### *Symptoms of deficiency*

Neither riboflavin nor pantothenic acid deficiency appears to produce characteristic gross symptoms in the duckling. On diets lacking either of these vitamins the birds fail to grow after the second or third day, and usually die within 4 to 7 days. There appears to be an excess of secretion from the

TABLE 1  
*Composition of the basal ration.*

BASAL MIXTURE		VITAMIN SUPPLEMENTS TO BASAL MIXTURE	
	%		$\mu\text{g}/100\text{ gm}$
Glucose .....	50.7	Thiamine chloride .....	400
Casein (vitamin-free) .....	18.0	Pyridoxine hydrochloride .....	400
Gelatin .....	10.0	Riboflavin .....	800
Corn oil .....	10.0	Calcium pantothenate .....	2500
Salt mixture (Hegsted et al., '41) ..	0.5	Nicotinic acid .....	4000
$\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ .....	1.0	Biotin .....	20
Cod liver oil .....	2.0	Folic acid .....	100
Cellu flour .....	3.0	Menadione .....	100
Choline chloride .....	0.3	Alpha tocopherol .....	50,000

eyes, and the eyelids may become stuck together. No evidence of curled toe paralysis or dermatitis, the characteristic symptoms of these deficiencies in the chick, has been noted.

The failure to show symptoms is probably not the result of the severe lack of vitamin in these experiments. The few birds which survive longer periods, and some which have been maintained on suboptimum levels of either of the vitamins, also fail to show characteristic signs. It may be noted that of all the vitamin deficiencies so far studied in ducklings, the only characteristic symptoms which have been observed are the polyneuritis of thiamine deficiency, paralysis in vitamin A

deficiency, and perosis in choline deficiency. Deficiencies of vitamins D, E, and K have as yet not been studied, and pyridoxine deficiency is the only deficiency disease which has been studied in chronic form. The occasional convulsion observed in pyridoxine deficiency is too rare to be considered typical of the disease.

### *Riboflavin requirements*

The data, collected in 3 experiments in each of which 5 levels of riboflavin were fed, were discussed extensively in a previous publication (Hegsted, '48) and need not be presented here. That publication also describes the methods used in evaluating the data. The values for the gain of each animal were plotted against the logarithm of the vitamin concentration in the diet. The regression line was then calculated through all of those groups in which the response appears to be approximately linear to the logarithm of the vitamin concentration. The minimum requirement for growth is taken as the point at which the regression line reaches the mean gain of the groups at the highest levels of intake. A minimum requirement for maintenance may also be calculated. This is the vitamin concentration which gives zero gain according to the regression line.

The results obtained for 3 experiments have been previously summarized (Hegsted, '48). It was concluded that the minimum requirement for growth is approximately 400  $\mu\text{g}$  of riboflavin per 100 gm of diet and that approximately 90  $\mu\text{g}$  per 100 gm is required for maintenance.

### *Pantothenic acid requirements*

Data on the pantothenic acid requirements were obtained in a similar manner in 2 experiments. Figure 1 shows the levels of pantothenic acid fed and the gain per day of each bird. The regression lines were calculated for all of the values from 200 to 1500  $\mu\text{g}$ , inclusive, of calcium pantothenate per 100 gm of ration. The mean requirement for maximum

gain was taken as the calcium pantothenate value required by the regression line to give a gain equal to the average of the 2 groups receiving the highest levels, and the maintenance requirement the amount to give zero gain. The values thus calculated are shown in table 2.

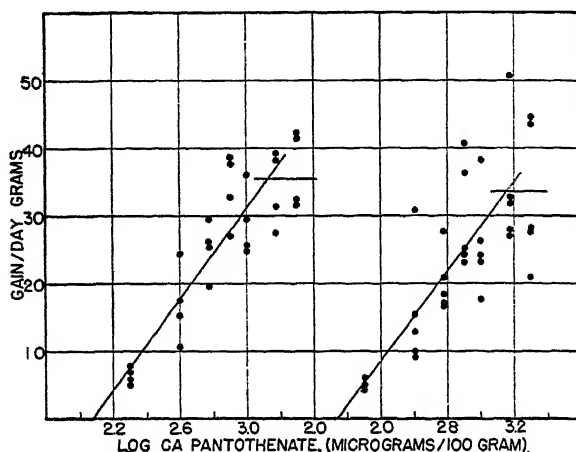


Fig.1 Scatter diagram showing the gain per day of ducklings which received diets with varying amounts of pantothenic acid. Regression lines were calculated as described in the text.

TABLE 2  
*Pantothenic acid requirements of ducklings.*

EXPT.	NUMBER OF BIRDS USED	AVERAGE MAXIMUM GAIN	CALCIUM PANTOTHENATE REQUIREMENT	
			Growth	Maintenance
		<i>gm/day</i>	<i>µg/100 gm</i>	<i>µg/100 gm</i>
1	28	35.4	1350	115
2	33	33.5	1415	133
		Mean	1382	124
		As pantothenic acid	1175	105

## DISCUSSION

It has been pointed out in a previous discussion (Hegsted, '48) that the method of treating the data may give higher

estimates of requirements for growth than the statistical comparison of the differences of the mean gains obtained for the various levels of vitamin concentration in the diets. This must be borne in mind in comparing these estimates with values in the literature since differences may be of interpretation rather than in the data themselves. However, only 1 paper on the riboflavin requirements of ducklings is available and none on pantothenic acid. Fritz, Archer and Barker ('39) estimated the minimum requirement to be 300  $\mu\text{g}$  of riboflavin per 100 gm of diet. This is in the same general range as previous estimates for chicks which have ranged from 200 to 350  $\mu\text{g}$  per 100 gm of diet (see review by Sherwood and Couch, '45). Lepkovsky and Jukes ('35) concluded that the chick requirement was about 230  $\mu\text{g}$  and that of poult was reported to be approximately the same (Jukes, '38).

The pantothenic acid requirements presented for chicks have varied even more than the estimates of riboflavin requirement. Jukes ('39) estimated it to be 1400  $\mu\text{g}$  per 100 gm of diet while Bauernfeind et al. ('42) concluded that 600  $\mu\text{g}$  per 100 gm was more nearly correct. Recent studies by Lepkovsky et al. ('45) suggest values between 460 and 970  $\mu\text{g}$  per 100 gm of diet for chicks and between 970 and 1170 for turkey poults. If the requirements of ducklings are similar to those of these species, our figures would favor the higher estimates.

Lepkovsky et al. ('45) suggest that requirements may vary with the rate of growth. While more carefully controlled studies are clearly needed to demonstrate this, it would appear to be highly probable. In this respect it is disconcerting to note that the maximum gain attained on the riboflavin studies was considerably below that observed in the studies on pantothenic acid. No explanation is available other than the possibility that this is due to a seasonal variation perhaps affecting the diets of the laying birds. It is of course possible that the riboflavin requirements would have been higher had maximum growth been obtained, but this seems unlikely in view of the fact that the estimated requirements are already above those reported for other species.

Although the practical significance of the maintenance requirement calculated in these studies is problematical, it is of interest to note that approximately 10 times the maintenance value of pantothenic acid was needed for maximum growth, whereas with riboflavin only 4 to 5 times the maintenance value was needed.

It is realized that requirements should probably be related to the composition of the diet. Fat (Mannering et al., '44) has been observed to increase riboflavin needs and high levels of protein appear to lower pantothenic acid requirements (Nelson et al., '47). However, the best estimates of requirements which can be made may be subject to errors great enough to obscure these effects under practical conditions.

#### CONCLUSION

Studies on the riboflavin and pantothenic acid requirements for growth of ducklings have yielded estimates of approximately 400  $\mu$ g of riboflavin and 1100  $\mu$ g of pantothenic acid per 100 gm of diet. Neither of these deficiencies produces characteristic symptoms in the duckling.

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# FALSE HIGH VALUES FOR ASCORBIC ACID IN GUAVA JUICE

A NOTE ON THE USE OF THE COLORIMETRIC METHOD  
WITH 2,4-DINITROPHENYLHYDRAZINE <sup>1</sup>

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(Received for publication December 22, 1947)

In the course of some studies on the stability of ascorbic acid in bottled guava juice the colorimetric method of Roe and Oesterling ('44) for total ascorbic acid was applied. The values obtained were much higher than those found with the usual dye-titration method. Samples which by the titration method showed complete loss of ascorbic acid gave values indicating practically no loss by the colorimetric method. In an effort to clarify this situation the following studies were carried out.

A number of additional samples of guava juice were assayed for reduced ascorbic acid by the dye-titration method and for total ascorbic acid by the colorimetric method during various periods of storage. The samples were assayed on the day the bottles were originally opened, then recapped and kept in the refrigerator during the experimental storage periods. The results are shown in table 1.

In every case the value obtained by the colorimetric method was as high as or higher than that obtained by dye-titration.

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Also the values obtained by the colorimetric method indicated little or no loss of ascorbic acid during the storage period. On the other hand, the values obtained by dye-titration indicated a rapid and considerable loss of the vitamin.

This discrepancy between the vitamin values obtained by the 2 methods might be accounted for on the basis of the presence of dehydroascorbic acid since only reduced ascorbic acid was determined by the dye-titration method. In order

TABLE 1

*Comparison of values for ascorbic acid in guava juice as determined by the dye-titration and colorimetric methods. All values are in mg/100 ml of juice.*

SAMPLE	WEEKS IN ICE BOX	METHOD	
		Dye titration	Colori- metric
5	0	60	71
5	1	57	71
5	3	20	73
5	5	0	69
6	0	60	65
6	1	58	65
6	3	37	68
6	5	3	61
8	0	69	75
8	5	6	81
8	8	0	77
9	5	13	83
9	11	0	79
9	15	0	65

to check on this point, 2 samples of guava juice were assayed by the dye-titration method both before and after reduction with  $H_2S$ , as well as by the colorimetric method. Assays were made on the 2 samples on the day the bottles were opened and after 3 and 4 weeks' storage in the refrigerator. The results are shown in table 2.

The data show that these samples did contain some dehydroascorbic acid as determined by the dye-titration method, but

the total ascorbic acid measured by this method was considerably less than that found by the colorimetric method. Also there was a loss of total ascorbic acid during storage as measured by the dye-titration method, whereas the colorimetric method indicated little or no loss.

TABLE 2

*Comparison of values for total ascorbic acid in guava juice as determined by the dye-titration and colorimetric methods. All values are in mg/100 ml of juice.*

WEEKS STORED	SAMPLE 1 <sup>1</sup>				SAMPLE 2 <sup>1</sup>			
	Dye-titration method			Colorimetric method	Dye-titration method			Colorimetric method
	Reduced	Total	Dehydro	Total	Reduced	Total	Dehydro	Total
0	27	29	2	37	61	64	3	77
3	20	24	4	30	24	41	17	79
4	16	23	7	34	5	27	22	78

<sup>1</sup> Sample 1 was 2 years old and sample 2 was 7 months old at the time the bottles were opened.

TABLE 3

*Comparison of values by the 2 methods for firm and for soft fruits.  
All values are in mg/100gm of fruit.*

SAMPLE		DYE-TITRATION METHOD			COLORIMETRIC METHOD
		Reduced	Total	Dehydro	Total
1	Firm	204	201	..	210
2	Firm	136	137	1	145
3	Firm	125	129	4	132
4	Soft	92	109	17	128
5	Soft	98	117	19	148
6	Soft	80	99	19	125

The same procedure was then applied to fresh ripe guavas, some of which were firm, unbruised fruit and others which were soft, overripe and slightly bruised. Three samples of firm and 3 of soft fruits were assayed. The results are shown in table 3.

In this case the 2 methods check reasonably well with fresh firm fruit, but with soft, bruised fruit the colorimetric method again gives high values. Apparently some substance occurs in old samples which reacts with the reagent in the colorimetric method but does not react with the dye. This unknown substance seems to be a derivative of ascorbic acid, for the values obtained with the colorimetric method agree fairly well with those yielded by the dye-titration method in the case of fresh samples. Also during the storage of juices the values have always been equal to or less than the original value, never significantly higher. It seemed possible, however, that the guava extracts contained some substance which prevented the quantitative reduction of dehydroascorbic acid by  $H_2S$  and that the colorimetric values were correct.

This point was checked by the use of a biological method. There were available at the time 4 subjects who had been maintained for 5 weeks on an ascorbic acid-low diet supplemented with 75 mg ascorbic acid per day. These subjects had been maintaining very constant levels of excretion of ascorbic acid for 3 weeks. Any change of intake of ascorbic acid would be expected to be very quickly reflected in the urinary excretion. Thus it should be possible to check the true ascorbic acid content of guava juice by using these subjects. They were, therefore, continued on the previous ascorbic acid-low basal diet, but given various experimental supplements. Subject 1, acting as a positive control, was given 90 mg of synthetic ascorbic acid per day instead of the previous 75 mg. Subjects 2 and 3 were given guava juice in amounts which, according to the dye-titration method, provided 75 mg of ascorbic acid per day, but which, according to the colorimetric method, provided 110 and 115 mg per day, respectively. Subject 4 was given daily supplements of a guava juice which provided 75 mg per day on the basis of the colorimetric method, but only 37 mg per day according to the dye-titration method. These new supplements were given daily over a period of 1 week and daily excretions of ascorbic

acid were determined throughout the week. The results are shown in table 4.

Subject 1 who received an additional 15 mg of synthetic ascorbic acid per day showed an increased average excretion of 7 mg per day. Of the 3 subjects (numbers 2, 3 and 4) who received their ascorbic acid in the form of guava juice, numbers 2 and 3 showed no change in excretion level and number 4 showed a sharp drop. This is exactly what would be expected on the basis of the assay values determined by the dye-titration method. On the basis of the colorimetric assay values, subjects 2 and 3 would be expected to show increased excretion values and subject 4 should show no change. These data,

TABLE 4

*Effect of a change of supplements on the urinary excretion of ascorbic acid.*

SUBJECT	INTAKE			AVERAGE EXCRETION			
	Previous	Experimental		Previous weeks			Experimental <sup>1</sup>
		Dye method	Colorimetric method	1	2	3	
	mg/day	mg/day	mg/day	mg/day	mg/day	mg/day	mg/day
1	75	90	...	27	24	26	33
2	75	75	110	21	21	22	22
3	75	75	115	19	21	19	18
4	75	37	75	36	34	33	16

<sup>1</sup> Last 3 days of the period.

therefore, clearly support the assay values obtained by dye-titration and show that the colorimetric method gave false high values for the ascorbic acid content of the samples of guava juice.

Pijoan and Gerjovich ('46) have reported a similar experience with orange juice and concluded that the false high values were due to the presence of diketogulonic acid, a derivative of ascorbic acid. This is the probable explanation for the apparently high ascorbic acid values found in guava juice since these values were always related to the original ascorbic acid content. The interference of reductones and other related compounds was ruled out by using the modifications of

the Roe and Kuether method described by Penney and Zilva ('45). These authors point out that 2,3-diketogulonic acid cannot be distinguished from l-ascorbic acid by this method.

Apparently the colorimetric method must be applied with caution particularly in the case of samples which have been stored. If the object is to determine the amount of ascorbic acid which was originally present, then the colorimetric method may be the method of choice.

#### SUMMARY AND CONCLUSIONS

Determinations of the ascorbic acid content of stored guava juice by the colorimetric method gave values which: (1) were considerably higher than those obtained by dye-titration and (2) indicated little or no loss of ascorbic acid during storage of the juice. Samples of guava juice given to human subjects who were receiving an ascorbic acid-low diet resulted in urinary excretion values which corresponded to the assay values obtained by dye-titration.

The colorimetric method, therefore, gave false high values for the ascorbic acid content of the stored guava juice.

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# THE EFFECT OF VITAMIN A DEFICIENCY UPON THE NITROGEN METABOLISM OF THE RAT

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## TWO FIGURES

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The role of vitamin A in metabolism remains obscure. In order to obtain data that might bear on this problem this experiment on the changes in nitrogen metabolism of vitamin A-deficient animals was undertaken.

Sampson, Dennison and Korenchevsky ('32) reported increased nitrogen metabolism and decreased rate of gain in body weight per gram of nitrogen ingested when rats were being depleted of vitamin A, as compared with pair-fed animals receiving vitamin A. Emerique ('37) found during avitaminosis A that the nitrogen balance remained positive although progressively smaller, and concluded that the failure of protein anabolism was the cause of the eventual loss of appetite. Braman et al. ('35) observed in a paired feeding experiment lasting 84 days that on a vitamin A-deficient diet, young rats gained less live weight and less nitrogen than their controls, but were not different from the latter with respect to digestion of the diets, nitrogen excretion, energy and fat production per gram of nitrogen retained. Only borderline deficiency was established in these rats so that the food intake of the deficient group was maintained at nearly normal levels.

Sure and Ford ('42), in an extensive study of the effect of 4 B vitamin deficiencies upon the urinary nitrogen partition

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in rats, found that the excretion of ammonia, allantoin, and creatinine was significantly changed on one or more of the deficient diets examined. No data as to food intake or nitrogen balance were reported.

#### METHOD

Several litters of similar age and body weight were divided at weaning, with the usual precautions, into 3 groups, and placed at once upon the diets as described later. The controls were fed the basal diet *ad libitum*, as were those given the vitamin A-free diet. The restricted controls which received vitamin A were given only the amount of basal diet eaten by the vitamin A-free group, but in proportion to the body weights.

Two series of experiments were made at different levels of protein content. In the first series the basal diet (diet I), consisted (in per cent) of vitamin-free casein 18, irradiated hydrogenated cottonseed oil 5, agar 2, salts <sup>2</sup> 4, cornstarch 71. In the second series the basal diet (diet II) contained in per cent vitamin-free casein 22, irradiated hydrogenated cottonseed oil 5, salts <sup>3</sup> 2.5, cornstarch 60.5, brewers' yeast 10. The B vitamins in the first series were supplied as rice bran concentrate <sup>4</sup> 0.1 gm and autoclaved yeast <sup>5</sup> 0.5 gm per rat per day. Cod liver oil providing 100 I.U. vitamin A per 100 gm rat per day was given the control and restricted groups in both series. Diet I contained 2.4 to 2.6% N and diet II, 3.4 to 3.5%.

In the first experiment both young and adult animals were used; in the second, only young rats. The young rats were placed on the experimental diet at 21 days of age and the adult animals at 85 days of age, when they weighed approxi-

<sup>2</sup> Osborne, T. B., and L. B. Mendel, *J. Biol. Chem.*, 45: 277, 1921.

<sup>3</sup> Hubbell, R. B., L. B. Mendel and A. J. Wakeman, *J. Nutrition*, 14: 273, 1937.

<sup>4</sup> This was the concentrate marketed as Galen "B" by the Galen Company, Inc., Berkeley, California.

<sup>5</sup> Northwestern brewery yeast autoclaved 8 hours at 15 to 18 lbs. pressure, and dried at 100°C.

mately 145 gm. The latter group, designated series IC, consisted of 20 rats from the same litters used in series IA, which were placed on the basal diet with full vitamin supplements at weaning and which, after 64 days were distributed among the 3 experimental regimes. Nitrogen balances and urinary nitrogen partitions were then recorded throughout the depletion and deficiency periods of 124 days. After 78 days, however, the deficient and restricted groups were each divided into 2 subgroups, the animals showing evidence of deficiency, series IC<sub>1</sub>, thus being separated from those which were not yet fully depleted, series IC<sub>2</sub>.

At the beginning and end of the experiments, representative rats of each group were fasted for 12 hours, to insure relatively empty gastrointestinal tracts, weighed, killed by gassing and the entire carcasses put through a grinder several times. Nitrogen, fat, water and ash analyses by the usual procedures were carried out immediately. The nitrogen content of the basal diet, supplements, urine and feces was determined by Kjeldahl procedure, upon aliquots of the pooled urine and washings and pooled feces of the different groups. The methods employed for the estimation of the various urinary constituents were Van Slyke manometric method ('32) for urea, the permutit and Nesslerization directions of Folin and Bell ('17) for ammonia, the method of Benedict and Franke ('22) for uric acid, Folin method ('14) for total and preformed creatinine, and, in most instances, the Handovsky modification ('14) of the Wiekowski method for allantoin. Some allantoin determinations in the first series were made by the Larson method ('32) and those in the second series were all made by the method of Young and Conway ('42).

## RESULTS

### *Growth*

The young rats on diet I grew less than those on diet II, possibly because of the lower protein and B vitamin contents of their diet, resulting in about 10% less food consumption.

The relative performances of the vitamin-deficient, restricted and control groups in the 2 experiments were similar, however, and are summarized together (table 1). The deficient young animals in both series had about 93% of the food and protein intake of the 2 fullfed controls but gained only 39% as much weight, while the restricted normal group also consumed 93% of the control intake but attained 74% of its weight increase. The decrease in weight gain was nearly directly proportional to decrease in intake in the restricted group, but was 2 to 3 times as great in the deficient group. This was similar to, but more striking than, the difference found by Braman et al. ('35) in mildly vitamin A-deficient rats.

The response of the older rats was different. During the 78 days of the preliminary depletion period, small gains were made by all the groups. In the latter portion of the period, when the depletion was evident, the pairfed rats had 70 and 74% and the deficient animals had 56 and 77% of the intake of the unrestricted controls, but both groups lost weight in about the same proportion. There seemed to be little difference between the restricted and deficient adult rats in utilization of calories and protein.

### *Carcass analyses*

The losses in body weight during the avitaminosis were accompanied by loss of fat from the carcass and increased content of water and, in the oldest rats, increased content of protein (table 2).

Representative carcasses were analyzed in experiment IA when the rats were 52 and 73 days old. The former group gave data on the body composition of the animals when the first failure of appetite occurred in the deficient group but before any other signs of depletion were seen, and the latter furnished information on the condition when the experiment was terminated after severe avitaminosis had been established. The body composition of all the 52-day-old rats was similar, but loss of fat and small increases in water were seen in the bodies of the 73-day-old deficient animals. Similar changes

TABLE 1  
*Gain per gram nitrogen intake of normal, paired and vitamin A-deficient young and adult rats.*

DIET	SERIES	AGES	NO. OF RATS	CONDITION	BODY WEIGHTS			N INTAKE		GAIN PER GM N EATEN
					Begin-ning	End	Gain	PERIOD	Daily per 100 gm wt.	Total
		days			gm	gm	gm	days	mg	gm
I	IA	36-74	11	Normal	71	159	88	38	220	9.6
			11	Paired <sup>1</sup>	64	129	65	38	205	7.4
			11	Deficient	59	93	34	38	205	5.9
II	II	31-61 or 65	11	Normal	59	165	106	30	344	11.8
			11	Paired	59	142	83	34	303	10.5
			14	Deficient	59	101	42	34	314	8.7
I	IC	85-164 <sup>2</sup>	6	Normal	146	198	52	78	125	15.8
			7	Paired	142	172	30	78	116	14.0
			6	Deficient	138	176	38	78	126	15.3
I	IC <sub>1</sub>	165-181	5	Normal	198	205	6	17	127	4.3
			3	Paired	171	156	-15	17	88	2.4
			3	Deficient	194	172	-22	17	71	2.1
I	IC <sub>2</sub>	165-240	3	Normal	224	249	25	46	144	16.5
			3	Paired	186	172	-14	46	106	8.4
			3	Deficient	175	153	-22	46	111	8.4

<sup>1</sup> In all cases intake of the paired groups was equalized per 100 gm body weight with that of the deficient group.

<sup>2</sup> On complete diets from weaning to 85 days of age.

were observed in the deficient young rats of experiment II. The adult rats, 178 days old when sacrificed, were found to diverge from the others in that those receiving vitamin A had considerably more fat and the deficient animals less fat than the corresponding young rats in the other 2 experiments. The nitrogen and ash contents of the deficient group were significantly greater than those of either the control or paired group.

TABLE 2

*Composition of the carcasses of normal, paired and vitamin A-deficient rats.*

SERIES	GROUP	AGE	NO. OF RATS	AVERAGE BODY WEIGHT	FAT (ETHER EXTRACT)	PRO-TEIN	WATER	ASH	REST
		<i>days</i>		<i>gm</i>	<i>%</i>	<i>%</i>	<i>%</i>	<i>%</i>	<i>%</i>
0	Normal	31	17	53	4.9	18.7	71.3	3.8	1.3
IA	Normal	52	4	111	6.1	21.3	67.3	3.8	1.5
	Paired	52	3	99	6.6	20.9	67.3	4.5	0.7
	Deficient	52	4	86	5.6	21.2	68.2	4.1	0.9
	Normal	73	4	146	7.4	21.1	66.6	3.9	1.0
	Paired	73	4	129	8.4	20.4	66.5	4.0	0.6
	Deficient	73	5	97	3.8	21.4	70.1	4.1	0.6
	Normal	178	5	208	17.7	18.3	61.1	4.2	1.3
	Paired	178	3	187	10.7	20.0	66.1	4.8	1.6
	Deficient	178	3	161	2.2	24.5	66.6	6.5	0.2
II	Normal	59	8	163	8.9	20.0	66.3	3.0	1.8
	Paired	63	9	147	7.4	20.2	65.6	3.6	3.2
	Deficient	63	11	95	2.6	20.8	69.0	3.1	4.5

The retention of food nitrogen in the carcasses of the 2 groups of young rats (table 3) indicates the same efficiency of transformation of food to body protein by the normal and paired rats on the 2 diets, and a lower utilization by the deficient rats in both cases, namely, 55 and 62% of the normal.

### *The nitrogen balances*

The nitrogen balances of the young deficient rats were progressively less positive as the deficiency increased in

severity. This was due to increased urinary output since there was little change in the fecal nitrogen other than that produced by changes in the intake. Results of some of the studies are shown in table 4. The nitrogen metabolism in all series was studied until some or all of the deficient group had succumbed. The average retention of the young rats on diet II was 30 to 50% greater in all cases than in the corresponding groups on diet I, but the retention of the paired group was significantly greater in both series than that of the deficient animals. The deficiency state reached by the rats in series IA was more severe than that shown by the animals in series

TABLE 3

*Retention of food nitrogen in the carcasses of normal, paired and vitamin A-deficient rats.*

SERIES	GROUP	AGE	NITROGEN CONTENT OF CARCASS			TOTAL NITROGEN INTAKE	RETENTION OF NITROGEN OF INTAKE
			At be- ginning	At end	Gain		
		<i>days</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>%</i>
IA	Normal	52-73	3.78	4.93	1.15	5.3	22
	Paired	52-73	3.31	4.21	0.90	4.1	22
	Deficient	52-73	2.91	3.31	0.40	3.3	12
II	Normal	31-59	1.77	5.12	3.35	11.5	29
	Paired	31-63	1.77	4.32	2.55	9.2	28
	Deficient	31-63	1.77	3.30	1.53	8.4	18

II, and the average nitrogen retention for series IA was also less.

No clear change in the nitrogen balance was observable in any of the adult deficient rats other than that simultaneously shown by their paired mates, even though all the deficient adult animals died of the deficiency. The rapidity of the onset of terminal changes appeared to influence the large nitrogen losses of series IC<sub>1</sub> as compared with IC<sub>2</sub>. Short periods of 8 and 6 days, in which the deficient and paired adult rats had exactly equal intakes, indicate the relatively small advantage in nitrogen retention of the rats which received vitamin A.

either to starvation or to the deficiency. In series IC<sub>1</sub>, representing the most advanced and in some cases premortal changes, a rise in the excretion of ammonia and allantoin and a larger drop in urea excretion were manifested by the vitamin-deficient as compared with the paired groups. This was not true of the more prolonged terminal stages shown in series IC<sub>2</sub>.

Ammonia nitrogen was found in somewhat increased amount only in the premortal excretion of the deficient and paired adult animals. The ammonia output remained at a low level, about 1% of total nitrogen, throughout all the balances on

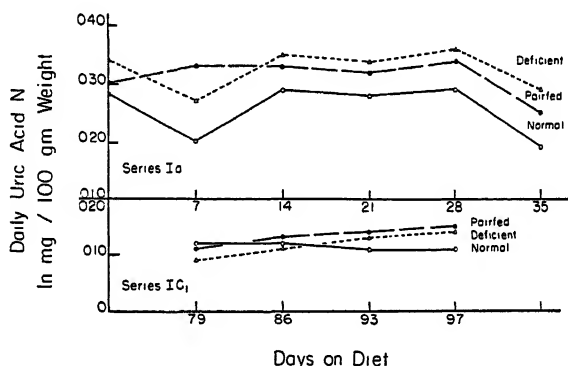


Fig. 1 The uric acid excretion of normal, paired and vitamin A-deficient young (series IA) and adult (series IC) rats.

diet I, but was increased to from 4 to 7% on the higher protein diet II. Urea was not determined in the latter series.

Preformed creatinine remained constant at about 1.0 mg per 100 gm body weight in all the groups, young and old. The creatine output was about twice as large in the young as in the older animals, in proportion to total nitrogen output. This is the usual result of decreased food intake.

The uric acid excretion was twice as great in the young as in the older animals but there was no change which could be ascribed to either starvation or the deficiency in any of the series, except for the slight premortal rise noted in both the deficient and paired groups of series IC<sub>1</sub> (fig. 1).

The allantoin excretion was also greater in proportion both to body weight and to total urinary nitrogen in the young than in the older rats. Both young and adult deficient animals excreted more allantoin than the paired or normal rats from the time that the deficiency became manifest, and particularly in the premortal period (fig. 2). This is not clearly shown in the daily averages compiled for the entire period of observa-

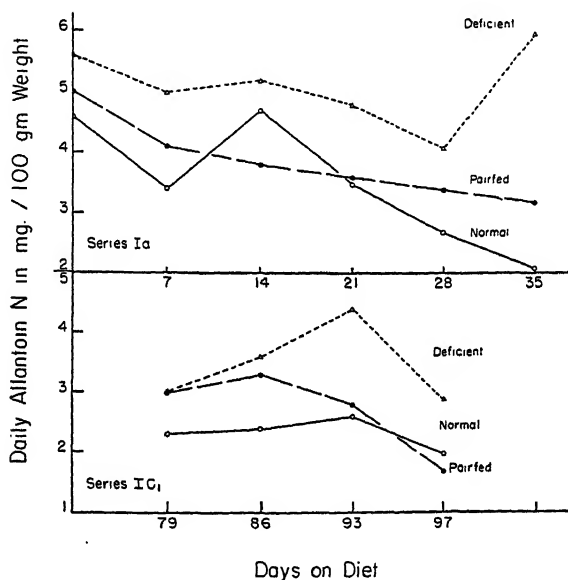


Fig. 2 The allantoin excretion of normal, paired and vitamin A-deficient young (series Ia) and adult (series IC<sub>1</sub>) rats.

tion, in which the premortal changes are masked. On diet II in which the deficiency was less severe than on diet I, the increased allantoin output of the deficient rats was not observed. This may be due to the more generous supply of necessary amino acids available, to the relatively mild deficiency, or to both of these factors. In several other experiments with the lower protein diet and severely deficient young animals the increased allantoin excretion was repeatedly observed. Sure and Ford ('42) reported only decreases in both



allantoin and uric acid in all their B vitamin-deficient rats, but again the values quoted were averages without separate report on terminal figures.

If allantoin and uric acid are products of essential tissue degradation, an accelerated rate of such breakdown occurred in both the vitamin A-deficient rats and their paired controls in the final stages of the deficiency. The greater excretion of allantoin by the deficient group might mean that purine or pyrimidine constituents of certain enzyme systems may become unusable in the absence of vitamin A and be discarded as allantoin.

### *Effect of level of dietary protein*

The young rats on diet II which received nearly 40% more protein than did those on diet I, grew more than the latter but utilized their food for growth less efficiently, whether or not they received vitamin A. The higher protein fed rats which received vitamin A stored considerably more fat than did those on the lower protein diet.

The nitrogen balances were also about 40% greater in the higher than in the lower protein-fed groups which received vitamin A but were much greater, about 150%, in the deficient animals. This was due to the fact that the urinary excretion of nitrogen was 50 to 80% greater in the normal and paired high protein groups but only 26% greater in the deficient animals, than in the corresponding lower protein-fed rats. It must be remembered, however, that the deficiency was more severe in the latter series.

The proportion of nitrogen excreted as uric acid and allantoin was not changed by the difference in level of protein intake, but the absolute and relative amount of urinary ammonia nitrogen was raised in the group fed the more protein-rich diet.

It appears that the catabolic processes of the protein metabolism were abnormally stimulated in the vitamin A-deficient growing organism, beyond that which might have been expected due to decreased food intake. The qualitative nature

of the resulting tissue breakdown or failure of tissue growth was not different from that produced by partial inanition alone, with the possible exception of the purine metabolism. This confirms the findings of Sampson and Korenchevsky ('32).

Since the nitrogen metabolism of the adult animals was not affected by the deficiency except in the premortal stage, it may be concluded that vitamin A is essential for tissue growth but not for maintenance.

#### SUMMARY

Young and adult rats from the same litters were divided into 3 groups, vitamin A-deficient, pairfed and fullfed on a diet containing 18% casein, and their nitrogen metabolism studied for 30 to 124 days. Another group of young rats was placed on a 22% casein diet and was similarly studied.

As the deficiency progressed, growth per gram of protein eaten declined in the deficient young rats to 65% of that of the pairfed groups. The urinary nitrogen increased and the nitrogen balance was decreased to a greater degree in the deficient than in the pairfed young animals. But in the adult group weight changes and nitrogen balances were alike in the pairfed and deficient rats.

The urinary nitrogen partitions were essentially alike in the pairfed and deficient groups except in the severely deficient and premortal periods when the allantoin output of the deficient animals was increased above that of the pairfed litter mates.

The composition of the carcasses of the fullfed, pairfed and deficient young rats remained constant except for the fat content which was diminished in the latter group with corresponding increase in water. As shown by carcass analysis, the retention of food nitrogen by the pairfed and fullfed young rats was the same in both series, but that of the deficient groups was only 55 and 62% of these values.

The deficiency state, as distinct from the accompanying inanition, decreased the utilization of nitrogen in the young

animals without affecting the character of the nitrogen metabolism except in the terminal period. The adult deficient and paired groups exhibited no differences in nitrogen metabolism. Vitamin A may therefore be considered essential for the growth of tissue protein but not for its maintenance.

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# NITROGEN METABOLISM OF THE NORMAL AND THE VITAMIN A-DEFICIENT RAT AS AFFECTED BY THYROID ADMINISTRATION

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## ONE FIGURE

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Possible interrelations between vitamin A and thyroid function have been the subject of numerous investigations in recent years. In a review, Drill ('43) summarized the status of the question as controversial.

Chevallier and Baert ('34) found that the basal metabolic rate of rats tended to rise on vitamin A depletion and to fall below normal when large doses were given. Sadhu and Brody ('47) have also observed the latter phenomenon. Abelin ('35) reported that vitamin A could reduce the high basal metabolic rate produced in rats given toxic doses of thyroxine. Belasco and Murlin ('40) found that vitamin A and ascorbic acid reduced to some extent the hypermetabolism associated with hyperthyroidism. Sheets and Struck ('42) saw no decrease in the metabolic rate of thyroid-treated rats due to administration of large doses of vitamin A.

That thyroxine increases the utilization or decreases the storage of vitamin A is the view held by a number of investigators, including Euler and Klusmann ('32) and Fasold and Peters ('33). However, Logaras and Drummond ('38) and Baumann and Moore ('39) came to the opposite conclusion.

Vitamin A deficiency has usually been found to produce hypertrophy of the thyroid although some investigators have reported no effect. Coplan and Sampson ('35) noted a sex difference in rats in that the deficiency caused thyroid hypertrophy in the female but atrophy in the male. That there is an increased need for vitamin A in hyperthyroidism has been reported by many, including Drill ('43), Belasco and Murlin ('40) and Sure and Buchanan ('37).

The fact that the relationship of the thyroid to vitamin A activity is not clear appeared to warrant the present study. Furthermore, it seemed worthwhile to investigate in more detail the hypothesis that the requirement of vitamin A is increased in hyperthyroidism. If this is true one might expect thyroid-treated animals on vitamin A-deficient diets to succumb to vitamin A deficiency readily or to be exceedingly sensitive to hyperthyroidism so that thyrotoxicosis would speedily become prominent and severe. No studies of this problem appear to have been made.

The nitrogen metabolism of vitamin A-deficient young rats previously examined (Brown and Morgan, '48) by the methods used in the present study had been found to be markedly influenced by the deficiency as well as by the accompanying reduction in food intake. The addition of hyperthyroidism to the deficiency as well as to the inanition state might be expected to produce a further disturbance of the nitrogen metabolism which should be cumulative, if there is neither antagonism nor coördination of the thyroid and vitamin A functions. The changes in nitrogen metabolism of normal and vitamin A-deficient rats with and without thyroid medication were therefore used as an index of the interrelation.

#### METHOD

The basal diet was that designated diet II in the previous report (Brown and Morgan, '48). This diet provided ample amounts of iodine. Cod liver oil, providing 100 I.U. of vitamin A per 100 gm body weight per day was given to the non-

deficient rats, and desiccated thyroid,<sup>1</sup> 100 mg. per 100 gm body weight per day administered by mouth to the hyperthyroid groups. Young rats from 20 litters were placed at weaning on the diet and were grouped so that weight, sex and litter origins were equally comparable in the 6 groups used. These groups were (1) normal, that is, receiving vitamin A and with no food restriction, (2) restricted or paired, receiving vitamin A but with food intake restricted to that of the deficient group in proportion to body weight, and (3) deficient, without vitamin A and not restricted as to food. Three additional groups on these regimes were given the desiccated thyroid. The males and females of each group were studied separately, so that in all 12 experimental groups were formed.

Soon after they were weaned, representative rats were sacrificed and moisture, ash, protein and fat determined in the carcasses. Similar analyses were carried out on all carcasses at the end of the experiments.

Urine and feces collections were made (a pooled sample for each group) for periods of 3 or 4 days consecutively for 34 to 38 days. Observations were made on the nitrogen balance, and on the excretion of ammonia, uric acid and allantoin. The methods employed were the same as those used previously (Brown and Morgan, '48), the allantoin being determined by the directions of Young and Conway ('42).

The experiments were terminated soon after all the rats of the groups restricted in intake and given thyroid had died. At this time the vitamin A-free groups were not as deficient as were the animals at the termination of studies in our earlier experiments. Less noticeable changes in all factors previously noted in vitamin A deficiency were therefore to be expected. No rats had demonstrated extreme avitaminosis and none had succumbed to the deficiency when it was uncomplicated by thyroid administration. Between the thirtieth and thirty-eighth day of the experiment, 4 males and 1 female

<sup>1</sup> Desiccated thyroid, U.S.P.

of the deficient group given thyroid had died, as had also 2 females and 1 male of the normal group given thyroid.

## RESULTS

### *Growth*

The growth of the groups is shown in figure 1. It will be noted that only in the thyroid-treated animals which were restricted as to food intake was there any consistent loss of weight during the period of the experiment. However, the gains made by the normal, pairfed and deficient groups of

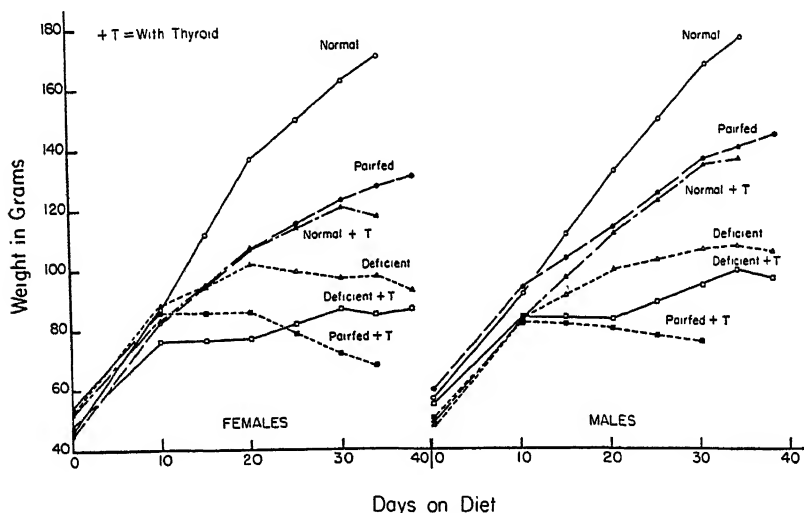


Fig. 1 The growth of normal, pairfed and vitamin A-deficient rats as affected by thyroid administration.

both sexes were always greater than those made by the corresponding hyperthyroid animals. There was only a small difference, however, between the deficient groups with and without thyroid.

The utilization of food nitrogen for growth (table 2) was decreased by the vitamin A deficiency as well as by thyroid treatment. It was evident that food restriction had little effect on protein utilization when vitamin A was present, but that the vitamin-deficiency markedly reduced utilization,

particularly in the females. The thyroid treatment only slightly affected the deficient females, but more seriously the deficient males. When these values are compared with the utilization by the normal groups either with or without thyroid, it is evident that the vitamin deficient rats were less affected by the thyroid treatment than were the normal unrestricted groups. In fact, the deficient females were benefited by the treatment when compared with the control thyroid-treated group, and the males were affected exactly as much as were their normal controls.

The most severe growth failures were seen in the food-restricted groups given thyroid.

So far as utilization of protein for growth is concerned, the amount of thyroid medication employed reduced by 54 and 36% the efficiency of normal well-fed female and male rats, by 16 and 33% that of vitamin A-deficient animals, and by 83 and 84% that of normal but food-restricted animals. In the female animals the deficiency apparently decreased the thyroid toxicity, and in the males it had little effect upon that condition. On the other hand, the thyroid seemed to nullify the deficiency effects in the females but to produce no change in the males. Food restriction alone affected utilization relatively little but depressed it severely when thyroid was given.

Administration of this amount of thyroid induced marked increases in food intake, while vitamin A deficiency alone caused somewhat decreased appetite. Vitamin A-deficient rats when given thyroid showed no inanition tendency, however, but ate as much or more than did the normal rats. An interesting finding here is that the usual and general trend of decreased intake per 100 gm weight as the animals became older did not hold either in the control group receiving thyroid or in the vitamin A-free rats given thyroid.

Sampson and coworkers ('32) reported a decrease in efficiency of food utilization for growth when rats were being depleted of vitamin A. This observation was verified in our results. Our hyperthyroid animals were likewise inefficient in



protein utilization. This was observed also by Koger, Hurst and Turner ('42).

### *Carcass analysis*

Since the nitrogen retention of the thyroid-treated rats was in some cases greater than that of the non-hyperthyroid animals, analysis of the carcasses seemed indicated. In table 1

TABLE 1

*Composition of the carcasses of normal, vitamin A-deficient and paired rats as affected by thyroid administration.*

GROUP		AGE	NO. OF CAR- CASES	AV. BODY WEIGHT	ETHER EX- TRACT	PROTEIN (N×6.25)	WATER	ASH	REST
		<i>days</i>		<i>gm</i>	%	%	%	%	%
Young, rep- resentatives of litters		31	17	56	4.9	18.7	71.3	3.8	1.3
Normal	♀	65	4	163	9.2	19.6	65.9	3.1	2.2
	♂	65	4	163	8.6	20.3	66.4	2.8	1.9
Normal with thyroid	♀	65	3	117	5.2	18.4	70.1	3.4	2.9
	♂	65	4	140	4.7	19.5	69.4	4.0	2.4
Deficient	♀	69	6	90	2.6	20.8	69.1	3.0	4.5
	♂	69	5	101	2.6	20.8	68.8	3.2	4.6
Deficient with thyroid	♀	69	5	87	3.6	20.3	70.2	3.6	2.3
	♂	69	4	98	3.1	20.7	68.2	3.8	4.2
Restricted	♀	69	5	136	7.3	19.8	65.5	3.0	4.4
	♂	69	4	159	7.4	20.5	65.7	4.2	2.2
Restricted with thyroid	♀	61	5	63	0.9	21.1	70.4	4.6	3.0
	♂	61	7	66	0.9	19.5	72.2	4.4	3.0

the results are shown. Both the vitamin A deficiency and the thyroid treatment separately reduced the body fat and increased its water content, but together had less effect. There was no definite change in any case in the protein content.

The proportion of moisture in the carcasses of normal rats decreases as the animal ages. Animals given thyroid and those deficient in vitamin A had moisture content resembling

that of young weanling rats, with little difference between males and females. Thyroid administration caused a more marked increase in moisture content of the tissues of both normal and paired animals than in those of the deficient animals.

By calculation from the composition of these carcasses and of those of representative young rats at 31 days of age, at the beginning of the experiment, the amount of food protein retained can be obtained (table 2). The retention of food nitrogen in the carcasses of the normal and restricted groups was nearly the same but in the vitamin A-deficient group it was only a little more than half as much. All of the thyroid-treated groups retained less food protein in the carcasses than did their controls. The hyperthyroid females on normal and deficient diets retained the same proportion of intake, but the hyperthyroid deficient males retained slightly less than the corresponding animals on the normal diet. The hyperthyroid animals which were restricted in intake retained only 5 or 6% of the intake. Thus, the thyroid treatment decreased the protein-building ability of the normal females by about 60%, but of the deficient females by only 23%, and of the paired females by 76%. In the deficient and also the control males the thyroid depressed tissue production by the same amount, 40%, but in the paired males by 82%.

Judged by the amount of retention of food nitrogen in the carcasses, the hyperthyroid condition affected all the males similarly, regardless of vitamin A status, but depressed the normal females more severely than the vitamin deficient females. The deficiency alone also reduced the protein retention in both sexes, but the combined hyperthyroid and deficiency states produced reduction only in the males. Insofar as the inanition effect could be judged, it appeared to produce no significant loss of efficiency, but combined with thyroid administration, the paired hyperthyroid animals being compared with normal hyperthyroid rats, it caused 50 and 70% loss of tissue formation. The greater depression of the growth of males than females produced by hyperthyroidism in the

TABLE 2

*Gain in weight, food intake and retention of nitrogen in carcasses of normal, paired and vitamin A-deficient rats as affected by thyroid administration.<sup>1</sup>*

GROUP	NO. OF RATS	BODY WEIGHTS			PERIOD	NITROGEN INTAKE		GAIN PER NITROGEN EATEN	NITROGEN CONTENT OF CARCASSES			FOOD N RETENTION IN CARCASSES
		At beginning	At end	Gain		Total	Per day		At beginning	At end	Gain	
		gm	gm	gm	days	gm	gm	gm	gm	gm	gm	%
Females												
Normal	6	46	171	125	34	13.7	0.40	9.1	1.38	5.37	3.99	29
Hypothyroid normal	7	52	118	66	34	15.5	0.46	4.2	1.56	3.47	1.91	12
Deficient	10	53	93	40	38	10.4	0.27	3.8	1.59	3.10	1.51	14
Hypothyroid deficient	6	48	87	39	38	12.0	0.31	3.2	1.44	2.83	1.39	11
Paired	6	45	131	86	38	11.0	0.30	7.8	1.35	4.15	2.80	25
Hypothyroid paired	7	54	68	14	34	10.5	0.31	1.3	1.62	2.30	0.68	6
Males												
Normal	5	55	177	122	34	14.4	0.42	8.4	1.65	5.75	4.10	28
Hypothyroid normal	6	49	137	88	34	16.2	0.48	5.4	1.47	4.27	2.80	17
Deficient	6	48	101	53	38	9.6	0.25	5.5	1.44	3.36	1.92	20
Hypothyroid deficient	8	55	97	42	38	12.7	0.33	3.7	1.65	3.21	1.56	12
Paired	5	57	145	88	38	11.0	0.30	8.0	1.71	4.76	3.05	28
Hypothyroid paired	10	50	63	13	30	9.6	0.32	1.3	1.50	1.97	0.47	5

<sup>1</sup> 100 mg dried thyroid per 100 gm body weight per day.

<sup>2</sup> At 31 days of age.

absence of vitamin A was also seen by Logaras and Drummond ('38).

### *Nitrogen retention*

The retentions of nitrogen, as measured by the balance method, by the normal and paired female rats during the later period of observation were alike, but that of the deficient group was much reduced. The deficient male rats under similar circumstances retained very slightly less nitrogen than their paired control groups (table 3).

TABLE 3

*The nitrogen metabolism of normal, vitamin A-deficient and paired rats with and without thyroid, from the nineteenth to the thirtieth day on the diet*

GROUP	AVERAGE WEIGHT	NITROGEN					RETENTION OF NITROGEN OF INTAKE
		Intake	Urinary	Fecal	Retained	Total re- tained	
Females	<i>gm</i>	<i>mg/100 gm body weight/day</i>					<i>%</i>
Normal	151	274	175	30	69	1.25	25
Normal with thyroid	114	443	292	58	93	1.27	21
Deficient	88	262	205	31	26	0.27	10
Deficient with thyroid	85	443	285	67	91	0.93	21
Paired	125	255	178	27	50	0.75	20
Paired with thyroid	75	333	294	58	—19	—0.17	—6
Males							
Normal	156	293	157	29	107	2.00	36
Normal with thyroid	127	418	249	78	91	1.39	22
Deficient	105	245	169	29	47	0.59	19
Deficient with thyroid	93	440	247	75	118	1.32	27
Paired	134	227	155	23	49	0.79	21
Paired with thyroid	82	291	306	65	—80	—0.79	—27

All the hyperthyroid rats without vitamin A had the same nitrogen intakes and the same or better relative retentions as compared with the corresponding groups which received the vitamin. It appeared that in both non-thyroid and thyroid-treated animals the vitamin deficiency had little deleterious influence on the nitrogen metabolism of males but depressed retention in non-thyroid treated females. The thyroid medication exerted a favorable effect in both sexes upon the nitro-

gen metabolism of the vitamin deficient animals. Thus, it is evident that both the thyroid administration and the vitamin deficiency state produced decreased nitrogen balances relative to intake, except when the 2 conditions were present together. Food restriction lowered the nitrogen retention in all cases, and combined with thyroid treatment led eventually to negative balances.

The thyroid-treated groups, both with and without vitamin A, were able to increase their intake sufficiently to maintain more positive nitrogen balances than the corresponding groups not receiving thyroid. Since the corresponding non-thyroid treated females, paired and normal, had more than double the nitrogen retention of the deficient group, one might well question that thyroid in the amount used and vitamin A are antagonistic in their effects. The larger nitrogen retentions in the deficient thyroid-treated groups may have been merely the result of the huge intakes. Yet, the fact that during the last several balances the hyperthyroid vitamin A-deficient groups retained as much nitrogen as the similar groups which received vitamin A is suggestive of a sparing effect due to the deficiency. The intake of these groups happened to be quite similar.

Johnston and Maroney ('39) with children as subjects found that administration of small doses of thyroid increased nitrogen retention while large doses reversed this effect. In severe hyperthyroidism positive nitrogen balances were obtained on high protein intake. Johnston ('41) reported that in girl patients resting in bed a small dose of thyroid stimulated appetite and allowed greater consumption of food so that the detrimental effect on nitrogen balance was avoided. Our rats reacted similarly to these human subjects.

Our results confirm also those of a limited experiment of Terroine and Babad ('39) using 4 rats. They reported that intraperitoneal injection of thyroxine into rats was followed by appetite increases so great that much more nitrogen was absorbed, but in spite of increased nitrogen retention there was a diminution in the rate of weight increase. In our ex-

periments the group of vitamin A-free animals given thyroid consumed larger quantities of food than either the deficient or normal animals; they retained more nitrogen in the latter part of the period yet did not attain as great weight. Similar comparisons can be made for the control groups with and without thyroid administration, where it will be noted that those which received thyroid during the period of observation ate much larger amounts of food and retained more nitrogen yet failed to gain as much weight as the control groups.

### *Urinary nitrogen partition*

Thyroid treatment during the latter part of the period when the vitamin deficiency was apparent, increased the urinary nitrogen in control, pairfed and deficient females by about the same amount, namely, 67, 65 and 61%, respectively, but in the males 59, 97 and 46%. The output of ammonia nitrogen in proportion to total urinary nitrogen was apparently not significantly affected by thyroid, although there was some tendency to an increase with thyroid treatment. The excretion of uric acid was strikingly constant in all groups in proportion to total nitrogen output. The proportion of allantoin nitrogen in the urine was lower in all thyroid-treated animals than in their controls, but this lowering was least pronounced in the deficient groups (table 4). None of these changes are of the magnitude found by Sure and his associates ('41) in a study of much more heavily thyroxine-treated young rats, but for the ammonia and allantoin at least, are in the same direction as the changes he reported.

The lack of vitamin A produced no appreciable change in the qualitative character of the nitrogen metabolism of our rats, insofar as this was indicated by the excretion of ammonia, uric acid and allantoin. \*

By the usual criteria of nitrogen metabolism, that is, utilization of food protein for growth and for production of tissue protein, amount of nitrogen retention, and urinary ammonia and purine output, the mild hyperthyroidism to which these

animals were subjected produced unfavorable effects in the normal fullfed and paired groups but much less severe depression in the vitamin A-deficient animals, especially the females. The vitamin A requirement was not increased in the hyperthyroid deficient groups, judged by these criteria, although the level of metabolism was obviously notably raised.

TABLE 4

*Daily average urinary nitrogen partition of normal, paired and vitamin A-deficient rats as affected by thyroid administration, from the nineteenth to thirtieth day on diet.*

GROUP	URINARY N	AMMONIA N		ALLANTOIN N		URIC ACID N	
Females	mg <sup>1</sup>	mg	% <sup>2</sup>	mg	%	mg	%
Normal	175	6.8	3.8	6.3	3.6	0.36	0.20
Normal with thyroid	292	13.2	4.5	7.9	2.8	0.59	0.21
Deficient	205	10.3	4.8	7.8	3.8	0.45	0.22
Deficient with thyroid	285	29.0	10.4	9.3	3.3	0.58	0.20
Paired	178	9.5	5.4	6.6	4.2	0.34	0.19
Paired with thyroid	294	15.8	5.3	9.6	3.3	0.57	0.19
Males							
Normal	157	5.9	3.6	5.9	3.8	0.33	0.21
Normal with thyroid	249	16.2	6.5	7.4	2.9	0.48	0.19
Deficient	169	13.4	8.4	6.7	4.1	0.36	0.22
Deficient with thyroid	247	23.0	9.5	8.1	3.6	0.53	0.22
Paired	155	12.1	7.5	6.4	4.3	0.32	0.21
Paired with thyroid	306	24.0	7.6	8.8	2.9	0.55	0.18

<sup>1</sup> In all cases per 100 gm body weight.

<sup>2</sup> Of total urinary nitrogen.

At the same time, since the body weights of all of these hyperthyroid animals were lower at all times than those of the untreated groups, the amount of vitamin A available in proportion to body weight must have been larger in the former than in the latter. This would support the assumption often put forth that vitamin A requirement is dependent upon body weight and independent of calorie output.

Since the marked decrease in nitrogen utilization caused by vitamin A deficiency in females was counterbalanced by the thyroid influence, it might be assumed that in the female the deficiency reduced thyroid activity, as suggested by the work of Coplan and Sampson ('35). No such counterbalancing by thyroid feeding was seen in the males, indicating possibly in this sex no specific effect of vitamin A deficiency on the thyroid gland.

#### SUMMARY

The nitrogen metabolism of young rats fed a purified diet containing 22% casein, with or without vitamin A and with or without administration of 100 mg desiccated thyroid per 100 gm body weight per day was observed for 30 to 38 days.

The utilization of nitrogen for growth was depressed by both the vitamin A deficiency and the thyroid treatment, more severely in the females than the males, but there was no cumulative effect when the 2 conditions were present together. Instead, the use of protein by the deficient animals, as shown by increase in carcass nitrogen and by growth, was less affected by the thyroid than was that of their normal controls.

Nitrogen retention during the later period of the deficiency was depressed by thyroid treatment in the fullfed and paired normal animals but improved in the deficient groups.

There was little change in the ammonia or uric acid fraction of the urinary nitrogen in any case, but a small decrease in allantoin excretion was noted in the hyperthyroid rats which was less pronounced in the deficient groups.

No increase in the vitamin A requirement resulted from this amount of thyroid treatment in either sex, but instead there may have been a reduction in the requirement of the females. No relationship is indicated therefore between total metabolism and vitamin A requirement as shown by nitrogen exchange.

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# THE ABSORPTION OF IRON FROM BEEF BY WOMEN

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Since the introduction of the theory that only a small proportion of the iron in the diet is absorbed, a number of experiments have been carried on to gather information on the actual percentage of iron which is absorbed under various conditions. Most of these studies have been carried out on animals. Human subjects on diets high in cereal content have been studied by Widdowson and McCance ('42), who found that when refined flour was used, women absorbed about 12.5% of the iron of the diet. The only other studies set up to find the percentage of iron absorbed by normal human subjects have been made with the use of radioactive iron. In these investigations iron salts rather than foods have been used. By this means normal men, children, pregnant women and normal women have been studied. The studies including normal women were made by Dubach et al. ('48) who found absorptions of from 1 to 10%. The present study was designed to contribute more information about the percentage of iron absorbed by normal women. At the same time material was collected for a study of the iron requirement which will be reported later.

The question of the method to employ was the first consideration. In recent years considerable experimental work has

accumulated which shows that iron is either not secreted or is secreted in only minute amounts from the blood plasma into the intestinal tract. Some iron may be added to the food-residue as it passes through the gastrointestinal tract due to sloughed-off cells and digestive secretions. The amount so picked up is not known; it may be even less than 0.1 mg and it is certainly never more than 0.5 mg. If the amount is assumed to be so small as to be negligible, the difference between the iron in the feces and that in the food gives the amount absorbed. This was the method employed by Widdowson and McCance ('42) whose main purpose was to compare refined and whole cereals rather than to obtain a value for the percentage of absorption. While the assumption may be valid, a method for determining absorption which would eliminate any effect of "picked-up" iron seemed preferable. The method decided upon was as follows: (1) To administer a basal diet containing an amount of iron just sufficient to cover the subject's needs; (2) to find the excretion in the feces on that diet; (3) to continue the basal diet and add the food to be tested; and (4) to determine the increase in intake due to the test-food, and the increase in fecal iron. The increases in the fecal iron would be due to the iron in the test-food that was not absorbed. The amount that was absorbed could be found by difference. This method is similar to one used by Kinsman et al. ('39) in the study of calcium absorption.

For the test-food, beef was chosen as it is used commonly in many diets and is high in iron. Furthermore, most of the previous work had indicated the iron of beef to be well absorbed. The earliest study on the absorption of iron from beef, one that did not agree with later work, and one in which rats were used as subjects, was made by Sherman, Elvehjem and Hart ('34) who concluded that the iron of beef was poorly absorbed. Later, Oldham ('41) tested cooked and uncooked beef and found that the iron of beef was as well absorbed as  $\text{FeCl}_3$  if the beef was cooked. Recently, Pye and MacLeod ('46) found that 32% of the iron of dried beef muscle was absorbed by growing rats. Oldham, Schlutz and Morse ('37)

found that the iron from beef was well absorbed by an infant. Leverton, who conducted a study on young women in 1941, found very high absorptions of beef-iron when the beef was fed after a long period on a depletion diet.

#### PROCEDURE

The subjects were 5 young women 18 years of age who were in excellent health. The weight (in pounds) and height (in inches) for subjects A, B, C, D and E, respectively, were as follows: 147 $\frac{1}{2}$  and 64 $\frac{1}{2}$ ; 139 $\frac{3}{4}$  and 63 $\frac{3}{4}$ ; 155 and 64 $\frac{1}{2}$ ; 138 $\frac{1}{2}$  and 64 $\frac{1}{2}$ ; and 148 and 68 $\frac{3}{4}$ . Subject E was 4 to 5 inches taller than the others.

On the basis of Leverton's ('41) observations, 7 mg of iron was selected as the lowest intake of iron which would be adequate to cover the needs of the subjects who were placed on a basal diet of that iron content for 10 weeks. The first 2 weeks constituted a period of adjustment during which some minor changes in the diet but no fecal collections were made. During the next 4 weeks the basal diet alone was fed and during the last 4 weeks ground beef ( $\frac{1}{3}$  fat,  $\frac{2}{3}$  lean meat) containing 3.4 mg of iron per day was added. This was served as 2 cakes of 100 gm each, 1 at breakfast and 1 at lunch. All the beef for the entire period was ground at 1 time, well mixed, made into 100 gm cakes and frozen.

The basal diet was adequate in all respects. It contained a quart of milk, an egg, 1 serving of meat, an average of more than 2 servings of vegetables a day besides potatoes and more than 2 servings of fruit a day. To avoid a high phytic acid content the diet contained no whole cereals, shelled beans, nuts or cocoa and only 1 serving of chocolate a week. It was calculated to contain 7.5 mg of iron, 1.2 mg thiamine, 70 gm protein, and 2400 calories. To hold the iron intake down to 7.5 mg necessitated the omission of only those foods that were extremely high in iron. The calcium, riboflavin and vitamin C were not calculated, as a large serving of citrus fruit every day insured sufficient vitamin C and a quart of milk insured sufficient calcium and riboflavin. The calculations

were made from the "Tables of Food Composition," prepared by the Bureau of Human Nutrition and Home Economics, and the National Research Council ('45) with the exception of the values for cooked meats taken from Bowes and Church ('46) and the value for the iron content of milk taken from Johnston ('44).

The basal diet was carefully controlled so that the intake for each week in the pre-beef period was exactly the same as that for the corresponding one in the beef period. The menu was planned on a weekly basis and was repeated so that it was the same on every succeeding corresponding day of the week. Paired cuts of meat were bought, 1 cooked immediately and 1 frozen for use on the corresponding day in the beef period. Both were cooked to the same internal temperature. All the fat was cut off and only lean meat weighed for the subjects. Canned goods were put up at 1 time in all-glass containers. All fresh and frozen vegetables and fruits were bought in 1 lot for the entire period. Distilled water was used for cooking, and drinking at meal time.

Foods allowed ad libitum were butter, sugar, coffee, a cola drink, tap water when away from the metabolic unit, and candy which was made from distilled water, sugar and other ingredients which were essentially iron free. Although these foods were fed ad libitum, a record was kept of the amount of each food eaten by each subject. The differences in the iron content of the diet in the pre-beef and beef periods due to variations in the amounts of these low iron foods were negligible. In a sense, the bread, rolls and biscuits were eaten ad libitum in that each subject decided the amount she desired during the 2 preliminary weeks and thereafter ate the same amount each week. An unenriched flour<sup>1</sup> was used so that the basal diet could be kept low in iron and at about the same level for all subjects.

<sup>1</sup> The flour was especially prepared for this project without enrichment by the Pillsbury Mills, Inc.

Care was taken to avoid any contamination during the preparation of the food. All cooking utensils were aluminum or glass and all knives were silver or stainless steel. Any places on the kitchen equipment which were susceptible to rust were coated with paint.

Weekly composites of the foods which all subjects ate in the same amounts were made in duplicate by different people. These were mixed in a Waring Blendor, treated with HCl, heated and stored in glass containers. The bread, biscuits, rolls, coffee, tap water and cola drinks were analyzed separately. For convenience the milk was analyzed separately although all subjects drank the same amount.

Carmine markers, for the separation of the stools, were given at the end of the 2-week adjustment period and at the end of each week thereafter. The weekly composites of the stools were digested with HCl and bottled.

The foods and stools were both wet-ashed with nitric and sulphuric acid, and the nitrosyl sulphuric acid hydrolyzed by boiling with water according to the method of Roberts, Beardsley and Taylor ('40). This method was deemed preferable to one employing perchloric acid as it was difficult to drive off all the perchloric acid in the samples of foods and stools taken after the addition of beef. The milk was dry-ashed in platinum dishes. The thiocyanate method of Stugart ('31) was used for the determination of iron. The colors were read in an Evelyn Photoelectric Colorimeter.

Four times, during the study, samples of venous blood were taken just before the noon meal and after a  $\frac{3}{4}$  hour rest period to eliminate the effect of exercise. Serum iron was determined on 2 ml samples. The protein was precipitated by the method of Kitzes, Elvehjem and Schuette ('44) and iron determined by the o-phenanthroline method of Saywell and Cunningham ('37). All determinations were made in duplicate. Hemoglobin was determined by the method of Evelyn ('36) and red blood cell counts were made in duplicate on blood from each of 2 pipettes.

## RESULTS

To find the absorption of iron from beef the following formula was applied:

$$\frac{\text{Iron in beef} - (\text{fecal iron on beef diet} - \text{fecal iron on basal diet})}{\text{Iron in beef}} \times 100$$

The iron absorptions for the 5 subjects were 32, 45, 58, 57, and 14% (table 1). Four of these women absorbed from  $\frac{1}{3}$  to over  $\frac{1}{2}$  the iron of the beef. Subject E, who was constipated much of the time, took mineral oil which caused a wide distribution of the carmine and made separation of feces between periods difficult. The stools of subjects A and B were normal while those of C and D were small and hard.

The retentions for subject E were especially high during the pre-beef period. This resulted in a paradoxical situation: according to the method of calculation used in this study, her percentage of absorption of beef iron was low, and yet on the beef diet she actually absorbed as much iron as subjects A and B.

Although this experiment was set up to offset the effect of iron "picked up" in the intestinal tract, absorptions may also be calculated on the diet as a whole if the amount of "picked-up" iron and iron secreted into the intestines is assumed to be negligible. In that case on the 7 mg daily intake, absorptions (food — feces) were 0.70, 0.32, 0.50, 0.69, and 1.40 mg or 10, 5, 7, 11 and 20% with a mean of 11% and on the 10.4 mg daily intake absorptions were 1.86, 1.85, 2.44, 2.61 and 1.84 mg or 18, 18, 23, 26 and 18% with a mean of 21%. Thus the percentage of iron absorbed (food — feces) was almost doubled after the addition of the beef.

The blood picture was one which showed all the subjects to be in good condition; they should have absorbed iron normally. Three of the subjects had serum iron levels of approximately 90  $\mu\text{g}$  per 100 ml, one had a level of the 100  $\mu\text{g}$ , and the other 121  $\mu\text{g}$ . These are normal levels for the method employed. If need is a factor which increases iron absorption, subject E whose serum iron was 121  $\mu\text{g}$  should have absorbed

TABLE I  
The retention of iron by 5 subjects from beef containing 3.4 mg per day.

IRON					IRON					IRON				
In food	In feces	Re-tained <sup>1</sup>	Retained from beef containing 3.4 mg Fe <sup>2</sup>		In food	In feces	Re-tained <sup>1</sup>	Retained from beef containing 3.4 mg Fe <sup>2</sup>		In food	In feces	Re-tained <sup>1</sup>	Retained from beef containing 3.4 mg Fe <sup>2</sup>	
mg/day	mg/day	mg/day	mg/day	%	mg/day	mg/day	mg/day	mg/day	%	mg/day	mg/day	mg/day	mg/day	%
Subject A														
Adjustment period:														
Week I	6.28				6.66					6.67				
Week II	6.68				6.85					6.89				
Basal period:														
Week III	6.87	6.42			6.85	5.98				7.02	6.26			
Week IV	6.94	5.65			7.09	7.94				7.10	6.52			
Week V	6.68	6.33			7.04	6.52				7.13	7.21			
Week VI	6.90	6.39			7.03	6.29				7.07	6.35			
Mean	6.90	6.20	0.70		7.00	6.68	0.32			7.08	6.58	0.50		
Beef period:														
Week VII	10.59	8.42			10.58	8.81				10.61	8.20			
Week VIII	10.37	7.58			10.52	7.98				10.54	7.85			
Week IX	10.41	9.23			10.45	8.75				10.59	7.61			
Week X	10.00	8.79			10.10	8.69				10.08	8.38			
Mean	10.36	8.50	1.86	1.10 32	10.41	8.56	1.85	1.52 45		10.45	8.01	2.44	1.97 58	
Subject D														
Adjustment period:														
Week I	6.13				6.68									
Week II	6.22				6.63									
Basal period:														
Week III	6.44	5.16			7.00	5.43								
Week IV	6.59	6.07			7.13	4.60								
Week V	6.52	6.00			7.08	5.30								
Week VI	6.56	6.12			7.07	7.37								
Mean	6.53	5.84	0.69		7.07	5.67	1.40							
Beef period:														
Week VII	10.07	6.49			10.61	7.93								
Week VIII	10.03	8.15			10.54	8.23								
Week IX	9.97	6.22			10.69	10.56								
Week X	9.54	8.32			10.08	7.72								
Mean	9.90	7.29	2.61	1.95 57	10.45	8.61	1.84	.46 14						

<sup>1</sup> Derived from the 2 preceding columns.

<sup>2</sup> Calculated from the formula given in the text.



less than the others. She actually absorbed more than the others on the basal diet. Probably all the subjects had good stores of iron and in no case was the "need" great enough to raise absorptions above normal levels. The fluctuations in serum iron from week to week were normal. There was no indication of a drop during the 6 weeks on the 7 mg diet, nor

TABLE 2

*The serum iron and hemoglobin values and red blood cell counts for the 5 subjects.*

SUBJECT	A	B	C	D	E
Iron in $\mu\text{g}$ per 100 ml of serum					
Feb. 17 — Diet started					
Feb. 22 — Adjustment period	..	116	82	100	115
Mar. 15 — Pre-beef period	83	74	104	88	119
Mar. 29 — Pre-beef period	116	80	97	103	119
Apr. 12 — Beef period	76	43 <sup>1</sup>	90	110	129
Mean	92	90	93	100	121
Hemoglobin in gm per 100 ml of blood					
Feb. 17 — Diet started					
Feb. 22 — Adjustment period	..	13.3	14.8	13.5	15.6
Mar. 8 — Pre-beef period <sup>2</sup>	13.7	13.6	14.0	14.0	14.9
Mar. 15 — Pre-beef period	13.0	12.8	13.3	14.0	14.9
Mar. 29 — Pre-beef period	13.2	13.7	13.9	13.3	15.4
Apr. 12 — Beef period	12.5	13.2	13.8	14.3	15.4
Mean	13.1	13.3	14.0	13.8	15.0
Red blood cell counts in $\bar{M}$ per $\text{mm}^3$					
Feb. 17 — Diet started					
Feb. 22 — Adjustment period	..	4.37	4.67	4.40	4.45
Mar. 15 — Pre-beef period	4.28	4.61	4.05	4.75	5.13
Mar. 29 — Pre-beef period	4.28	4.93	5.52	4.81	4.92
Apr. 12 — Beef period	4.22	4.99	5.36	5.08	4.57
Mean	4.26	4.73	4.90	4.76	4.79

<sup>1</sup> Severe sore throat. This value was omitted in computing the mean.

<sup>2</sup> Capillary blood.

of a rise within 2 weeks after the beef was added. The hemoglobin concentrations for all 5 subjects were up to or above 13 gm (table 2), which is considered a satisfactory level for women. The red cell count for all the subjects except one was up to 4.5  $\bar{M}$  per  $\text{mm}^3$  (table 2) which is considered an average count for women. The exception was a count of 4.26  $\bar{M}$  per  $\text{mm}^3$  which is high enough to be satisfactory.

It may be noted that the diet which was calculated to contain 7.5 mg of iron was found by analysis to contain 7.0 mg (table 1). From this, one might conclude that when a diet is free from any sources of iron contamination and when the tables of food composition used in this study are employed, calculation yields values which are sufficiently accurate for many purposes.

#### DISCUSSION

Although this experiment was set up to determine the percentage of iron absorbed from beef, the results can be interpreted in another way: possibly the increase in absorption after the beef was added was not all due to iron present in the beef itself but to some effect of the beef on the food mixture so that there was an increase in the amount of iron absorbed from the other foods in the diet. Beef may be reducing in effect, changing the ferric iron to the ferrous form which is more readily absorbed. Tompsett ('40) has shown that at the pH of the stomach ferric iron is reduced by certain protein foods. Mirsky and Anson ('36) have shown that sulfhydryl groups in muscle proteins can reduce iron and, also, that tyrosine and tryptophane have some reducing properties although less than those of the sulfhydryl groups. Meat is a good source of pteroylglutamic acid (P.G.A.) and of Castle's extrinsic factor. It is possible that these substances and other functionally related ones aid in the absorption of iron. Mims, Swendseid and Bird ('47) found that sulfhydryl groups inactivated certain inhibitors of P.G.A. conjugase. Thus meat may not only supply P.G.A. but, also, compounds which allow it to be used.

If one assumes that all the increase in the iron retention after the addition of the beef came from the beef, the absorptions from beef found in this study would be high and the conditions of the experiment should be examined to see whether or not they are higher than could be expected for other women. These women were all 18 years of age. It is possible that they were still growing at a slow rate. The ex-

periment of Darby et al. ('47) with radioactive iron on children shows that growth increases the amount of iron absorbed. Leverton and Marsh ('42) believe that iron absorption is better on an adequate diet such as the one used in this study than on one which is deficient in one or more respects. In all 5 subjects the food-residue remained in the intestines for a rather long period of time. In most cases it remained  $2\frac{1}{2}$  to 3 days, 4 times it remained  $4\frac{1}{2}$  days, and for subject E it sometimes remained as long as  $5\frac{1}{2}$  days. Since the absorption of iron normally occurs in the duodenum and upper jejunum this circumstance probably did not increase absorption. There is little likelihood that men would absorb as much iron as did these young women because their needs are not as great.

The mean value of 11% found in this study for absorption from the basal diet, if "picked-up" iron and iron secreted into the intestinal tract are considered negligible, is similar to that of 12.5% found by Widdowson and McCance ('42) with women subjects, and to the top values found by Dubach et al. ('48) using radioactive iron. The sudden increase in absorption of iron after the addition of beef found in this study is similar to the sudden increase found by Leverton ('41) whose subjects were in fecal balance on 4-5 mg of iron and whose retentions jumped almost 3 mg upon the addition of 116 gm of beef. The behavior of Leverton's subjects was more extreme than in the case of these subjects. The former had been on a basal diet of lower iron content continued for a longer time than in the present case, previous to the addition of beef.

In planning this study it was decided that periods of at least 4 weeks should be used. Had periods of 2 weeks been used, the values for C and D would have been the same as for the 4 weeks, but the values for the 3 other subjects were definitely altered by the use of the longer period. A 5- or 6-week period might have increased the accuracy of the results, but it is questionable whether the added accuracy would have justified the added trouble and expense. In this experiment, as in all balance experiments, there was variation in fecal losses from week to week. There is no way of knowing how much of this

variation is due to changes in absorption and how much is due to errors in separation of the stools into periods. These "errors" are unavoidable as the food from each meal does not stay in a separate compartment as it travels through the gastrointestinal tract. The only way to minimize them is to use long metabolic periods.

#### SUMMARY

Five young women 18 years of age, in good health, with normal serum iron, hemoglobin and red cell counts were placed on a diet containing 7 mg of iron which was judged to be the lowest intake that would be adequate. For 2 weeks minor adjustments were made in the diet and the subjects were accustomed to a 7 mg level. During the next 8 weeks the adjusted basal diet was administered. During the last 4 of the 8 weeks 2 100-gm patties of ground beef ( $\frac{2}{3}$  lean meat and  $\frac{1}{3}$  fat) containing 3.4 mg of iron were added, 1 at breakfast and 1 at lunch. During the 4-week basal period and the 4-week beef period, weekly collections of feces were made and during the entire 10 weeks, weekly composites of the foods were preserved for analysis.

When the formula:

$$\frac{\text{Iron in beef} - (\text{fecal iron on beef diet} - \text{fecal iron on basal diet})}{\text{Iron in beef}} \times 100$$

was applied the absorptions from beef for 4 out of 5 subjects were 32, 45, 58 and 57%. This led to the conclusion that young women 18 years of age on a diet adequate in all respects absorb from about  $\frac{1}{3}$  to more than  $\frac{1}{2}$  of the iron of beef.

Another interpretation of the results is more likely the correct one: that the beef improved the absorption of the iron from the entire food mixture, rather than itself contributing all of the iron absorbed after it was added to the diet.

If the amount of iron added to the food residue as it passes through the gastrointestinal tract can be regarded as negligible, the mean absorption from the basal diet containing 7

mg of iron was 11% and was increased to 21% after the addition of beef to the 2 meals not already containing meat.

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# THE EFFECT OF INANITION ON MAMMARY-GLAND DEVELOPMENT AND LACTATION <sup>1</sup>

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## TWO FIGURES

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Several investigators (Marrian and Parkes, '29; Mason and Wolfe, '30; Moore and Samuels, '31; Pomerantz and Mulinos, '39; Werner, '39, and Mulinos and Pomerantz, '40) have presented data which suggest that inanition causes a pituitary insufficiency. Mulinos and Pomerantz ('40) have designated the condition produced by inanition as pseudo-hypophysectomy because of the similarity of the effects of inanition and of hypophysectomy on various endocrine organs. Therefore, because inanition affects the endocrine organs, it is not surprising that it should retard the development of the mammary gland. This retarding effect has been noted by many workers (Astwood, Geschickter and Rausch, '37; Nathanson, Shaw and Franseen, '39; Trentin and Turner, '41; Huseby and Ball, '45). All these studies on mammary growth have involved the use of non-pregnant animals and, although Trentin and Turner ('41) have shown that the effectiveness of estrogen in inducing mammary growth parallels food intake, no data are available as to whether the estrogen produced during pregnancy would overcome the effect of inanition.

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The present experiment was undertaken, therefore, to determine the effect of inanition on mammary growth when feed restriction was imposed from weaning through pregnancy and to correlate mammary growth with subsequent lactation.

#### PROCEDURE

One hundred and twenty female white rats weaned at 25 to 26 days of age were divided into 2 groups of 60 each, with equal numbers of litter mates in both groups. They were housed 10 rats to a cage.

TABLE 1  
*Composition of diets.*

	STOCK DIET (CONTROL)	MODIFIED STOCK DIET (RESTRICTED)
	<i>lb.</i>	<i>lb.</i>
Corn meal	69.5	52.5
Linseed meal	14.0	19.0
Meat scrap	9.0	12.6
Casein	4.0	10.0
Alfalfa-leaf meal	2.0	2.8
Poultry bone meal	2.0	2.8
Salt	0.5	0.7

Group 1 (controls) were fed a stock diet in such amounts that minimal quantities of feed remained in the feed cups each morning. They can be considered to have been fed *ad libitum*. Group 2 animals (restricted intake) were fed the same diet as that fed to group 1, but the total amount of the diet fed was only 70% of that fed to group 1. The ingredients were so adjusted that, although the energy intake of these rats was reduced by 30%, they received the same amounts of protein and other food nutrients as the animals in group 1. The composition of the diets is shown in table 1. Lettuce and raw carrots were also fed once weekly. This stock diet supplemented with lettuce and carrots permits good growth, reproduction, and lactation in our colony.

All rats were mated at 100 days of age. At this time the number of females was reduced to 5 per cage, and the males were left with them for 14 days and alternated between cages at the end of 7 days. No further opportunity for mating was afforded.

At parturition, half of the rats in each lot were sacrificed. The 3 posterior mammary glands on the left side were dissected out, after removing and stretching an adequate area of hide together with the glands. The thyroid, adrenal, and pituitary glands were also obtained. Weights of the glands were determined. It was not possible to determine comparative degrees of mammary development by histological examination in these rats and while it was recognized that varying amounts of other tissue, particularly fat, between the 2 groups might seriously interfere with the validity of deductions based on weight data, this was the only quantitative measure at our disposal.

The remaining rats of both groups which bore young were allowed to suckle a litter of 12 for 21 days. Litter weights were obtained every other day during this period. The gain in weight of the litter was used as a measure of the lactation ability of the mother. All mother rats, whether or not they had been restricted in food intake prior to parturition, were placed on the stock diet during the nursing period and fed *ad libitum*. At the end of the nursing period the mother rats were killed and the weights of the mammary glands determined, as was done with the rats that were sacrificed at parturition.

Those rats that did not become pregnant were disposed of as soon as it was certain that they were not carrying young. They were killed at an age, therefore, which on the average was only slightly greater than that of the rats sacrificed at parturition. Mammary, thyroid, adrenal, and pituitary glands were obtained from these animals. In these non-pregnant rats, microscopic examination was the only means by which the comparative mammary development of rats on the control and restricted diets could be determined, since the limits of

the area of glandular development were so poorly defined that an accurate dissection was impossible.

## RESULTS

### *Mammary development*

Data on mammary-gland weights are shown in table 2. The average weight of the dissected mammary glands from rats that were killed at parturition was 3.34 gm for those on the control diet and 1.87 gm for those on the restricted diet. Glands from the control group were thus 80% heavier than those from the restricted group. The body weights of these 2 groups

TABLE 2

*The effect of reduced feed intake on mammary-gland development.*

	NUMBER OF RATS	AVERAGE WEIGHT OF MAMMARY GLAND	AVERAGE BODY WEIGHT AT AUTOPSY
		gm	gm
Group 1 (control) <sup>1</sup>	24	3.34	230.8
Group 2 (restricted) <sup>1</sup>	19	1.87	188.3
Group 1 (control) <sup>2</sup>	23	3.55	238.5
Group 2 (restricted to parturition) <sup>2</sup>	18	3.51	220.4

<sup>1</sup> Size of glands determined on day of parturition.

<sup>2</sup> Size of glands determined following a 21-day lactation period.

at autopsy averaged 230.8 and 188.3 gm, respectively, a difference of only 22.6%. While these values do not represent differences in the amount of secreting tissue alone, it is our opinion that they do represent comparative development. The exact magnitude of development, however, is unknown. That the values do not represent apparent differences which might in reality be an accompaniment of reduced body size or deposition of fat, or both, would appear to be indicated by the marked difference in gland weight (80.0%) as compared with the relatively small difference in body size (22.6%) of the 2 groups. The differences noted in mammary-gland weight were undoubtedly due in part to the relative amounts of fat de-

posited in the area of the gland; but even if one assumes that the amount of fat deposited in this area is less than in other parts of the body when animals are on a restricted dietary intake, the disproportion between gland size and body size was of sufficient magnitude as to make it highly improbable that a difference in fat deposition would by itself account for the effects noted. Variation in the amount of secretion present in the tissues, at the time they were removed and weighed, was kept to a minimum by leaving the litters with the mothers until just before autopsy. The 2 illustrations (figs. 1 and 2)

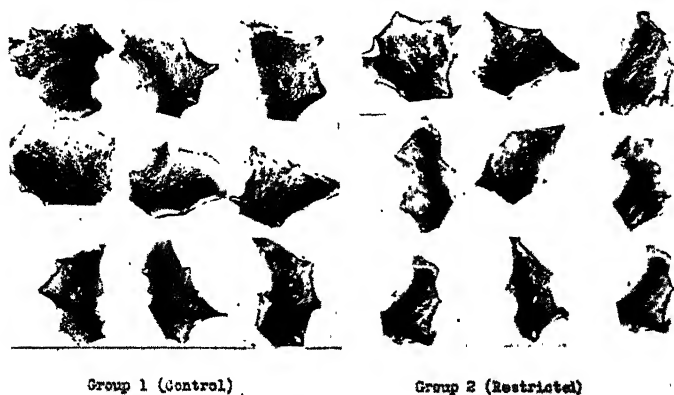


Fig. 1 Rat mammary glands obtained at parturition.

also indicate that there was a real difference in the mammary development of the 2 groups.

The individual glands shown in figure 1 were obtained from rats which bore young and were sacrificed at parturition. The glands were stretched on sheet cork, fixed in Bouin's fixative, washed and taken into 70% alcohol. The abdominal gland was separated as accurately as possible from the 2 smaller, more compact inguinal glands and photographs of the former were made with transmitted light. The glands shown in figure 1 are representative of the type of development observed. The upper 6 glands from the control rats are most

representative of the development seen in this group, while the lower 6 glands from the restricted rats represent the type of development observed in 80% of the rats on restricted intake. While differences in the density of glandular tissue are not marked, the extent of development is obviously greater in the control rats.

The glands shown in figure 2 were obtained from non-pregnant rats which were killed at approximately the same age as those whose glands are shown in figure 1. The gland on

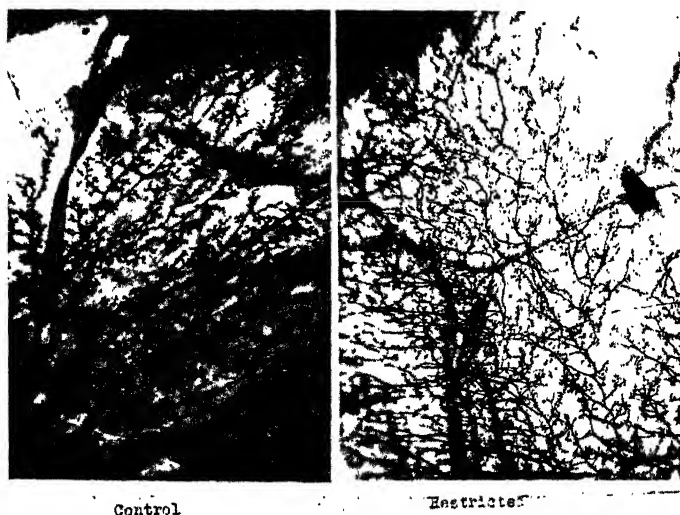


Fig. 2 Mammary glands from non-pregnant rats.

the right is one of the most highly developed glands observed in the non-pregnant restricted animals, while that on the left is representative of the average development of the control rats.

These results would therefore indicate that inanition reduces the development of mammary tissue in rats which become pregnant as well as in non-pregnant animals (Huseby and Ball, '45) or animals (Trentin and Turner, '41) in which mammary growth has been stimulated by estrogenic hormones. The retardation of development at parturition in our

animals would appear to be quantitative rather than qualitative. The glands at this time appeared mature in every respect in both the restricted and the control rats and, as will be shown, were capable of reasonably adequate lactation in both lots.

Whether or not this reduction in mammary growth was brought about indirectly through the effects of inanition on the endocrine system, as might be suggested from the work of Mulinos and Pomerantz ('40), is not clear from our data. The average weights of certain endocrine glands from restricted and control rats are given in table 3.

TABLE 3

*Comparison of endocrine glands of rats on full feed and on restricted diet.*

ANIMAL GROUP	WEIGHT OF		
	Pituitary	Adrenal	Thyroid
	mg	mg	mg
Killed at parturition			
Rats on full diet	11.8	51.7	23.0
Rats on restricted diet	9.9	45.2	17.6
Non-pregnant animals			
Rats on full diet	13.2 <sup>1</sup>	45.5 <sup>1</sup>	27.0
Rats on restricted diet	8.7	34.2	22.7

<sup>1</sup> The difference in weight was greater than might be expected from the difference in body weight in these 2 instances only.

The weights of the pituitary, adrenal, and thyroid glands were smaller throughout in the restricted rats but, except in the case of the pituitary and adrenal glands of the non-pregnant rats, this reduction in size was proportional to the reduction in body size. There may therefore have been some hormone deficiency in the non-pregnant (restricted) rats or in all the restricted rats up to the time when pregnancy occurred, if it is permissible to draw such a conclusion by using this disproportionality between gland weights and body weight as a basis. That this may have been so is also suggested by the slightly delayed time of vaginal opening and fewer number of pregnancies observed in the restricted rats. Ball,

Barnes and Visscher ('47) have noted a similar reduction of pregnancies in underfed mice. This deficiency, if present, appeared to disappear during pregnancy as judged by the fact that the endocrine gland weights were proportional to body weight at parturition. We were unable to demonstrate a difference in prolactin content of the pituitary glands which were removed at parturition. It would seem, therefore, that a hormone deficiency was not a major factor in the reduced mammary development observed at parturition.

When rats, which were underfed until parturition, were fed ad libitum during a 21-day lactation period, they gained in body weight rapidly until at the end of lactation they had attained body weights nearly equal to those of rats on full feed from weaning. A similar rapid increase in body weight was noted by Ball et al. ('47) when feed consumption was increased in their previously underfed mice. The average weights of the mammary glands had also become equal in the 2 groups. These data are also shown in table 2. During this period it would appear that a much more rapid growth of mammary tissue occurred than was the case with the other tissues. There was no apparent gain in the mammary tissue in group 1 during this period.

### *Lactation*

In spite of the fact that less mammary tissue was present at parturition in the restricted rats, these mothers raised superior litters. During this time they were fed ad libitum in order to eliminate the effect of reduced feed intake on lactation. Their feed consumption, as compared with that of the controls, must actually have been greater in relation to body size, since they gained in body weight. The gain per litter, the average weight of the young, and the survival of the young at the fourteenth and twenty-first days of lactation were greater in every instance in the case of mothers which had been subjected to reduced feed intake. These data are presented in table 4.

These animals, then, not only increased the amount of secreting tissue during this period, as shown in table 2, but they also secreted milk at a better rate than rats whose mammary glands had attained mature size at parturition. Possibly, the explanation for this difference in lactation performance is that regression of the functional activity of the mammary tissue may have set in relatively early during lactation in those glands that were fully developed at parturition (glands from rats of group 1). This regression in function apparently did not occur, or it was reduced in extent in the glands of the previously restricted rats (group 2) which were actively growing during the lactation period and were therefore presumably in a more viable condition. The similarity of the

TABLE 4

*The effect of inanition on lactation of rats during growth and pregnancy.*

	AVERAGE WEIGHT PER LITTER AT START	AT 14 DAYS			AT 21 DAYS		
		Total gain per litter	Average weight of young	Sur- viving	Total gain per litter	Average weight of young	Sur- viving
		gm	gm	%	gm	gm	%
Group 1 (control)	68.4	139.1	18.0	96.4	163.8	23.9	76.5
Group 2 (restricted)	63.6	154.5	18.4	98.7	198.3	25.3	86.0

effects of inanition on lactation obtained in this study with those obtained on reproduction by Ball, Barnes and Visscher ('47) is rather striking.

The degree of inanition which was imposed on these animals was not particularly severe. Reduction in body size occurred and the reproductive functions were apparently somewhat subnormal. The animals, however, appeared healthy at all times; there was no mortality, and they rapidly attained mature body size during lactation when adequate feed was available. In animals which are to be used for milk production, it may therefore be desirable to regulate food intake so that only moderate body size and mammary-gland development are attained at the time of first parturition, in order to attain the best results in the subsequent lactation periods.



## SUMMARY

Inanition in rats from weaning through pregnancy reduces growth of the mammary gland to a greater extent than might be expected from the reduction in body size which results from reduced feed intake.

When adequate feed was supplied during lactation to rats that had been on the restricted diet, they increased in body size rapidly, the mammary gland also grew rapidly, and their lactation performance was superior to that of rats that were on adequate feed intake throughout the whole of the experimental period.

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# CYSTINE AND METHIONINE METABOLISM BY CHICKS RECEIVING RAW OR AUTOCLAVED SOYBEAN OIL MEAL<sup>1</sup>

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Autoclaving soybean oil meal affects the nutritive value in 2 ways. Moderate autoclaving (such as at 100°C.-120°C. for 30 minutes or equivalent) has been shown to increase the nutritive value of raw soybean oil meal for growing chicks (Hayward et al., '37; Almquist et al., '42; Bird and Burkhardt, '43; Parsons, '43; Evans and McGinnis, '46), whereas a more drastic autoclaving, such as at 130°C. for 60 minutes, has been shown to decrease the nutritive value of the moderately autoclaved meal (Bird and Burkhardt, '43; Parsons, '43; Evans and McGinnis, '46; Clandinin et al., '47). The lowering of the nutritive value of proteins caused by heat-treatment has been recognized for several years (McCollum et al., '39) but only recently has it been shown that the nutritive value of soybean proteins is also injured by heat-treatment. The influence of heating on the soybean proteins is generally believed to be related to the methionine availability and metabolism (Melnick et al., '46).

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The present investigation was a study of the biochemical changes occurring in soybean oil meal proteins as a result of autoclaving and of the effects of these changes on cystine and methionine metabolism by the chick. Earlier work from this laboratory (Evans and McGinnis, '46) indicated that autoclaving selectively affected the availability of methionine, moderate autoclaving (100°–120°C. for 30 minutes) increasing methionine retention, and drastic autoclaving (130°C. for 30 or 60 minutes) decreasing it. This early work was not conclusive since gelatin and dried brewers' yeast furnished 40% of the dietary protein. In the present study soybean oil meal was the only source of dietary protein. The earlier work indicated that the cystine availability was also affected. Since no supplemental cystine was fed, it was not possible to determine the extent to which cystine could replace methionine as a supplement to the raw or over-autoclaved soybean oil meals.

#### EXPERIMENTAL

Groups of 20 New Hampshire chicks were fed a diet containing: cerelese 43.9 gm, soybean oil meal 48.0 gm, minerals <sup>3</sup> 5.0 gm, soybean oil 2.5 gm, fortified fish oil 0.5 gm, liver paste 0.1 gm, choline chloride 200 mg, tocopherol concentrate 10 mg, p-aminobenzoic acid 10 mg, nicotinic acid 3 mg, 2-methyl-1,4-naphthoquinone 1 mg, riboflavin 1 mg, calcium pantothenate 1 mg, thiamine 0.5 mg, pyridoxine 0.5 mg, pteroylglutamic acid 0.05 mg, and biotin 0.01 mg. The soybean oil meals represented 3 different types: (1) raw meal, (2) raw meal autoclaved for 30 minutes at 100°C. (this might be better referred to as a steaming treatment since little pressure was used), and (3) raw meal autoclaved for 60 minutes at 130°C. Groups of chicks fed the above soybean oil meals also received supplements of 0.5% L-cystine, 0.5% DL-methionine, and 0.5% cystine + 0.5% methionine. Chicks fed the more drastically auto-

<sup>3</sup> The composition of the mineral mixture was: oyster shell, 1570 gm; CaHPO<sub>4</sub> · 2H<sub>2</sub>O, 1740 gm; K<sub>2</sub>HPO<sub>4</sub>, 840 gm; NaCl, 600 gm; MgSO<sub>4</sub> · 7H<sub>2</sub>O, 500 gm; Fe<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub> · xH<sub>2</sub>O, 55 gm; MnSO<sub>4</sub> · 4H<sub>2</sub>O, 29 gm; KI, 3.3 gm; CaSO<sub>4</sub> · 5H<sub>2</sub>O, 1.5 gm; ZnCl<sub>2</sub>, 1.0 gm; and CoCl<sub>2</sub> · 6H<sub>2</sub>O, 0.2 gm.

claved meal also received supplements of 0.5% L-lysine and a combination of 0.5% cystine + 0.5% methionine + 0.5% lysine.

Sulfur balance studies were conducted with each group of chicks for 3 days during the third week of the experiment. All of the chicks in a group were considered as a single unit for the balance study. Cystine, methionine, and sulfate sulfur intakes were determined from the dietary feed intake and composition. Droppings were collected on glass plates and rapidly air-dried. Cystine, methionine, and sulfate sulfur were determined on the feed and the droppings by the differential oxidation procedure (Evans, '45). Excretion and retention of these were calculated.

Methionine, cystine, and lysine determinations were also made on acid and enzyme digests of the 3 soybean oil meals by microbiological procedures. Acid hydrolysates were prepared by autoclaving 1-gm samples in sealed tubes with 10 ml of 10% hydrochloric acid, diluting, neutralizing, and making to a suitable volume. *In vitro* trypsin-erepsin digests were prepared as described by Evans ('46). Lysine was assayed microbiologically, using *Streptococcus faecalis* R (Baumgarten et al., '46), and methionine and cystine were assayed with *Lactobacillus arabinosus* 17-5 (Riesen et al., '46). Amino nitrogen was determined by the method of Van Slyke ('13).

#### RESULTS

Cystine and methionine balance data for the chicks fed the unsupplemented soybean oil meals are presented in table 1. Over 50% of the ingested cystine was excreted in the droppings unchanged. The percentage of the ingested cystine sulfur which was not excreted unchanged is reported as cystine sulfur retained plus that excreted as sulfate. This value was obtained by subtracting the cystine sulfur excreted unchanged from the cystine sulfur ingested. It was not possible to determine how much of the "retained plus excreted as sulfate" cystine sulfur was retained for building body proteins and how much was oxidized to sulfate and excreted

as such. Similar calculations were made with the methionine data.

Data are also presented in table 1 for the cystine plus methionine sulfur excreted unchanged, cystine plus methionine sulfur oxidized to sulfate and excreted, and cystine plus methi-

TABLE 1

*Cystine and methionine balance of growing chicks fed raw and autoclaved soybean oil meals.*

	NOT AUTOCLAVED	AUTOCLAVED AT 100°C. FOR 30 MIN.	AUTOCLAVED AT 130°C. FOR 60 MIN.	AUTOCLAVED AT 130°C. FOR 60 MIN. + LYSINE
<i>Cystine sulfur</i>				
Intake (gm)	0.80	2.03	1.54	1.35
Excreted as cystine (gm)	0.62	1.07	1.07	0.91
Excreted as cystine (%) <sup>1</sup>	78	53	70	67
Retained + excreted as sulfate (%) <sup>1,2</sup>	22	47	30	33
<i>Methionine sulfur</i>				
Intake (gm)	0.36	0.92	0.70	0.62
Excreted as methionine (gm)	0.15	0.15	0.26	0.32
Excreted as methionine (%)	42	16	37	52
Retained + excreted as sulfate (%) <sup>2</sup>	58	84	63	48
<i>Cystine + methionine sulfur</i>				
Intake (gm)	1.16	2.94	2.23	1.96
Excreted as cystine and methionine (gm)	0.78	1.22	1.33	1.23
Excreted as cystine and methionine (%)	67	42	60	63
Retained + excreted as sulfate (%) <sup>2</sup>	33	58	40	37
Oxidized to sulfate (gm)	0.11	0.24	0.13	0.06
Oxidized to sulfate (%)	10	8	6	3
Retained (%)	23	50	34	34
Protein efficiency (chicks) <sup>3</sup>	1.4	2.1	1.5	1.5

<sup>1</sup> Per cent of total cystine intake.

<sup>2</sup> This value represents the percentage of total cystine or/and methionine in the soybean oil meal which was not excreted unchanged. Part of this was retained for building body proteins and part was oxidized to sulfate and excreted as such. See text.

<sup>3</sup> Grams gain in weight per gram of protein consumed.

onine sulfur retained. The latter value was determined by subtracting the sulfur excreted from the total sulfur intake. It was not possible to determine the proportions of the sulfur retention representing the sulfur in cystine and methionine, respectively, because of the inability to distinguish between the sulfate formed from cystine and that from methionine.

Moderate autoclaving, or steaming (100°C. for 30 min.), increased both the retention of cystine plus methionine sulfur by growing chicks and the protein efficiency, the latter being determined by the gain per gram of protein consumed (table 1). Both cystine and methionine were retained or oxidized to sulfate before excretion to a greater extent when the soybean oil meal was steamed.

The methionine plus cystine sulfur of the drastically autoclaved soybean oil meal (130°C. for 60 minutes) was retained by growing chicks to a lesser extent than that of the steamed meal, but to a slightly greater extent than that of the raw meal.

The addition of 0.5% lysine to the over-autoclaved soybean oil meal did not improve cystine plus methionine retention or protein efficiency.

Data concerning the metabolism of supplemental cystine and methionine are presented in table 2. For calculating these data it was assumed that the same percentages of the cystine and methionine contained in the soybean oil meal were excreted unchanged, retained, and oxidized to sulfate in the same manner as when no supplement was added. Any increases in these values were considered to come from the supplement.

About 30% of the supplementary cystine was retained by the chicks fed the raw and drastically autoclaved soybean oil meals when 0.5% L-cystine was added to the diet. Less was retained by the chicks fed the steamed meal. About 50% of the supplemental cystine was oxidized to sulfate and excreted as such, and 10 to 30% was excreted unchanged. Of a 0.5% methionine supplement, more than 50% was retained and 92% retained and metabolized by the chicks fed the raw soybean

TABLE 2

*The metabolism of supplementary cystine and methionine by growing chicks fed raw and autoclaved soybean oil meals.*

	NOT AUTOCLAVED	AUTOCLAVED AT 100°C. FOR 30 MIN.	AUTOCLAVED AT 130°C. FOR 60 MIN.	AUTOCLAVED AT 130°C. FOR 60 MIN. + LYSINE
	% <sup>1</sup>	% <sup>1</sup>	% <sup>1</sup>	% <sup>1</sup>
<i>Supplement — 0.5% cystine</i>				
Excreted as cystine	10	27	20	
Excreted as methionine	3	0	5	
Retained + excreted as sulfate	87	73	75	
Excreted as sulfate	56	55	45	
Retained	31	18	30	
Increased protein efficiency <sup>2</sup>	7	10	0	
<i>Supplement — 0.5% methionine</i>				
Excreted as methionine	5	3	14	
Excreted as cystine	1	5	9	
Retained + excreted as sulfate	94	92	77	
Excreted as sulfate	38	53	57	
Retained	56	34	20	
Increased protein efficiency <sup>2</sup>	29	5	0	
<i>Supplement — 0.5% cystine + 0.5% methionine</i>				
Cystine excreted as cystine	23	24	1	17
Cystine retained + excreted as sulfate	77	76	99	83
Methionine excreted as methionine	2	3	26	28
Methionine retained + excreted as sulfate	98	97	74	72
<i>Cystine + methionine:</i>				
Excreted unoxidized	14	15	13	22
Retained + excreted as sulfate	86	85	87	78
Excreted as sulfate	57	52	41	37
Retained	29	33	46	41
Increased protein efficiency <sup>2</sup>	14	5	7	33

<sup>1</sup> Per cent of supplemental cystine, methionine, or cystine + methionine consumed.

<sup>2</sup> The increased protein efficiency represents the percentage increase in protein efficiency resulting from the supplement or supplements added. For example, the protein efficiency for the raw meal was 1.4 and for the raw meal supplemented with 0.5% DL-methionine 1.8, an increase of  $\frac{0.4}{1.4} \times 100 = 29\%$ .

oil meal. This is in contrast to the 20% retained and 77% retained and metabolized by the chicks receiving the drastically autoclaved meal. Thirty-eight to 58% of the supplementary methionine was converted to sulfate and then excreted (metabolized).

Chicks fed a diet containing the drastically autoclaved soybean oil meal supplemented with both cystine and methionine retained and metabolized practically all of the supplemental

TABLE 3

*The influence of autoclaving soybean oil meal on the liberation of methionine, cystine and lysine by acid and by in vitro trypsin and erepsin digestion.*

METHOD OF HYDROLYSIS		NOT AUTOCLAVED		AUTOCLAVED 30 MIN. AT 100°C.		AUTOCLAVED 60 MIN. AT 180°C.	
		In soybean oil meal	Enzyme/ Acid	In soybean oil meal	Enzyme/ Acid	In soybean oil meal	Enzyme/ Acid
		%	%	%	%	%	%
Amino nitrogen	Acid	5.50		5.48		5.30	
	Enzyme	1.65	30	2.28	42	1.77	33
Methionine	Acid	0.71		0.71		0.75	
	Enzyme	0.35	49	0.50	70	0.40	53
Cystine	Acid	0.95		0.98		0.58	
Lysine	Acid	3.0		3.1		2.2	
	Enzyme	0.9	30	1.1	35	0.1	5

cystine but only 74% of the supplemental methionine compared to a retention and metabolism of 76% of the supplemental cystine and 98% of the supplemental methionine by the chicks fed the other 2 meals. The addition of 0.5% L-lysine did not improve cystine or methionine utilization or retention.

The results of the amino acid determinations on acid and enzyme digests of the soybean oil meals are presented in table 3. Steaming did not influence the methionine, cystine, or lysine contents. It did, however, increase the amounts liberated by enzyme digestion. More drastic autoclaving did not change the methionine content of the meal but destroyed 40% of the cystine and 30% of the lysine as determined by bacterio-



logical assay. There was a decreased liberation of methionine by enzymic digestion, which paralleled the decreased liberation of amino groups. Very little of the lysine was liberated by enzymic digestion. Cystine determinations on the enzymic digests could not be made because cystine peptides exert a greater stimulating action on *L. arabinosus* than does free cystine.

#### DISCUSSION

It is generally believed that raw soybean oil meal is deficient only in methionine for the growing chick. The data presented in this paper indicate that such is not the case but that raw soybean oil meal is deficient in essential amino acids other than methionine. Methionine additions to the raw soybean oil meal did not increase growth and protein efficiency (body weight gain of chicks per gram of protein consumed) to the extent that mild autoclaving did (McGinnis and Evans, '47). The data of Almquist et al. ('42) and Evans and McGinnis ('46) indicating only a methionine deficiency in raw soybean oil meal were obtained with diets containing considerable gelatin. Gelatin contains only low levels of cystine and methionine, but is a fair source of some of the other amino acids (Mitchell and Block, '46). Since methionine is the most limiting amino acid in soybean oil meal (Mitchell and Block, '46), methionine additions to the diet improved growth and protein utilization, but did not give maximum growth.

The present data can be explained by the poor digestion by the chick of the proteins of raw soybean oil meal (Evans, McGinnis and St. John, '47). A trypsin inhibitor has been shown to be present in raw soybean oil meal (Ham and Sandstedt, '44; Bowman, '44). When this inhibitor was added to diets containing autoclaved soybean oil meal, fish and meat meals, or casein, the protein nutritive value was decreased for chicks (Ham et al., '45) and rats (Klose et al., '46). This trypsin inhibitor is destroyed by heat.

Drastic autoclaving of soybean oil meal decreases the protein nutritive value by decreasing the availability of the lysine, and the methionine or cystine or both, since the nutritive value

can be restored to optimal by supplementing with 0.34% DL-methionine and 0.65% L-lysine monohydrochloride (Clandinin et al., '47), or with 0.5% DL-methionine, 0.5% L-cystine, and 0.5% L-lysine (McGinnis and Evans, '47). Parsons ('43) earlier observed that drastic autoclaving (123°C. for 90 minutes) decreased the protein nutritive value of soybeans for rats and that supplementation with 0.2% D-lysine partially corrected this. The lysine of soybean oil meal was partially destroyed by the drastic autoclaving. Of the remaining lysine, part was converted to a form which was not liberated from the proteins by enzymic digestion as shown by Riesen et al. ('47) and by the present studies.

Drastic autoclaving of soybean oil meal destroyed part of the cystine, according to the microbiological assays, but not according to the differential oxidation procedure. This destruction may have been by racemization or by conversion to some readily oxidized, biologically inactive compound. The destruction was of sufficient magnitude to explain the increased excretion (biologically active or inactive) and the decreased retention and metabolism of cystine by the chick.

No mechanism for the selective inactivation of methionine by drastic autoclaving has been advanced. No destruction of methionine, as shown by microbiological assay on the acid-digested meal, was observed by Riesen et al. ('47) or in the present study. Russell et al. ('46) observed that much of the methionine in certain legumes was not available to the growing rat, but could offer no reason for this.

The microbiological data in table 3 show that drastic autoclaving of the soybean oil meal decreased the percentage of methionine which was liberated by *in vitro* trypsin and erepsin digestion. Evans, McGinnis and St. John ('47) observed a "selective concentration" of methionine in the protein fraction of drastically autoclaved soybean oil meal not digested by the chick. Their data show that 6.7% of the methionine sulfur of the steamed meal was in the undigested protein fraction while 25.6% of that of the drastically autoclaved meal was in this fraction, an increase of 3.8 times. The same

data show that 11.5% of the protein of the steamed soybean oil meal was not digested, compared with 16.1% of the protein of the drastically autoclaved meal, or an increase of 1.6 times.

The data of table 2 indicate that metabolism of the supplemental methionine by the chick was interfered with when the diet contained the drastically autoclaved soybean oil meal. Retention of supplementary methionine by the chick was reduced from 33% on the steamed soybean oil meal to 20% on the drastically autoclaved meal. Chicks fed the raw meal retained 54% of the supplemental methionine. It appears that autoclaving soybean oil meal at 130°C. for 60 minutes may have caused the formation in the meal of some substance or substances which interfered with the normal absorption or metabolism, or both, of methionine. Part of the methionine, both that present in the soybean oil meal and that in the supplement, was apparently "tied up" in such a manner that it could not be utilized for tissue building. Considerable methionine was excreted unchanged (possibly unabsorbed) and part was oxidized to sulfate and excreted.

#### SUMMARY

Mild autoclaving, or steaming (100°C. for 30 minutes), of a raw soybean oil meal increased the percentage of the cystine and methionine present in the meal that was retained or metabolized by the growing chick. Steaming apparently increased the digestibility of the soybean oil meal proteins, probably by a destruction of the trypsin inhibitor.

More drastic autoclaving (130°C. for 60 minutes) of a raw soybean oil meal reduced the percentage of the cystine and methionine present in the meal that was retained or metabolized as compared to the steamed meal.

Retention of supplemental L-cystine by the chicks fed the drastically autoclaved soybean oil meal was the same as that for the chicks fed the raw meal. Retention of the supplemental DL-methionine was, however, decreased from 56% of the intake for chicks fed the raw meal to 20% for those fed the drastically autoclaved meal, and supplemental methionine excretion

was increased from 3% to 26%. The formation, by drastic autoclaving, of a substance in the soybean oil meal which interfered with normal digestion and metabolism of methionine is indicated. The addition of supplemental L-lysine to the diet did not affect the cystine and methionine metabolism.

Autoclaving did not influence the methionine content of soybean oil meal as determined microbiologically. Drastic autoclaving destroyed 40% of the cystine as shown by a similar assay.

The liberation of methionine from soybean oil meal by *in vitro* digestion with trypsin and erepsin was increased by steaming but again decreased by drastic autoclaving.

The drastic autoclaving procedure reduced the nutritive value of soybean oil meal by (1) a partial destruction of cystine and lysine, (2) a decreased digestibility of the lysine not destroyed, and (3) a decreased absorption and utilization of the methionine, probably caused by the formation of a substance which interfered with normal digestion and metabolism of methionine.

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# THE EROSIVE ACTION OF VARIOUS FRUIT JUICES ON THE LOWER MOLAR TEETH OF THE ALBINO RAT<sup>1</sup>

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The erosive action of a dilute solution of hydrochloric acid, grapefruit juice and cranberry juice cocktail on the molar teeth of the albino rat (McClure, '43) suggests that similar effects might be expected from any other acid-containing beverage. From the dietetic point of view this is a matter of considerable significance because of the acidic nature of many fruit juices that have an extensive commercial distribution, and inferentially have become fairly common articles of diet. The present study was undertaken to obtain comparative experimental data on the action of a number of these juices on the teeth.

The experiment consisted of 2 parts. In the first part, the juices served as the sole source of drinking fluid for the test animals; in the second, the juices were administered 3 times a day in measured amounts and constituted only a portion of the fluid intake.

## PROCEDURE

### *Part 1. Fruit juices constituting the entire fluid intake*

Weanling rats were selected from the colony and placed in individual cages. Thenceforth throughout the duration of the

<sup>1</sup> The expense of this investigation was defrayed in large part by a grant-in-aid from the Sugar Research Foundation.

experiment extending over 100 days, they were fed a commercial dog food <sup>2</sup> exclusively which they were allowed to eat *ad libitum*. The sole source of fluid of each test animal during this time was one of the following juices: apple, grape, orange, tomato, unsweetened grapefruit to which 10 gm sugar per 100 ml was added, pineapple and prune. Sugar was added to the grapefruit juice because the animals would not drink it unsweetened. These juices, all of which were canned, were purchased at random from the shelves of a neighborhood grocery store with no consideration given to the trade name. The juices that contained pulp were centrifuged and only the supernatant fluid free from solid material was used. The control animals were allowed to drink water *ad libitum*.

The daily consumption of the various juices by the weanling rats increased rapidly day by day and amounted to 35 ml within less than 2 weeks. Thereafter the daily intake was restricted to this amount. This was somewhat in excess of the daily water intake of the controls which amounted to an average of 26 ml. In the course of the experiment each test animal drank a total of 3.5 liters of fruit juice. It was considered advisable to limit the intake of the juices to a definite amount because the rat is not temperate in its drinking habits when given a fluid that appeals to its taste. Unless restricted, the daily consumption of a delectable fluid will exceed by several times the normal intake of water.

At the conclusion of the experiment the animals were decapitated. After boiling the heads in water for 30 minutes the teeth were removed, dried and weighed. They were then studied under the microscope and the degree of erosion evaluated by following in general the criteria of Restarski, Gortner and McCay ('45). High polish of lingual enamel, slight etching, mild destruction, moderate destruction and severe destruction were graded, respectively, 1, 2, 3, 4 and 5. The teeth were then redried and weighed and the percentage enamel determined by the procedure of Manly and Hodge ('39). This method does not yield absolutely accurate results, as a few of

<sup>2</sup> Purina dog chow.

the enamel particles cling to the dentine in the separation of the 2 fractions. However, it suffices for a relatively good degree of quantitative approximation for a comparative evaluation of erosion.

### RESULTS

The data of the experiment on the lower molars are presented in table 1. Similar data were obtained on the upper as on the lower molars, but these are omitted from the table for the reason that the uppers showed very little erosion except for those of the test group on grapefruit juice. In no

TABLE 1

*Effects of various juices on the lower molar teeth of the white rat when consumed as the sole drinking fluid for 100 days.*

FLUID CONSUMED	WEIGHT OF LOWER MOLARS	LOSS IN WEIGHT	DEGREE OF ERO- SION <sup>1</sup>	WEIGHT OF ENAMEL	LOSS IN WEIGHT OF ENAMEL	TITRA- TABLE ACID	pH	NUMBER OF ANIMALS
	mg	%		mg	%	%		
Water	69.3	.	0	15.2	...	.	..	36
Tomato juice	64.5	6.9	1	12.7	16.4	0.37	4.2	9
Prune juice	65.2	5.9	1	12.2	19.7	0.54	3.6	7
Orange juice	63.3	8.6	3	11.8	22.4	1.08	3.6	8
Pineapple juice	64.1	7.5	2	11.6	23.7	0.78	3.4	12
Apple juice	62.4	10.0	4	11.4	25.0	0.54	3.5	10
Grape juice	61.5	11.3	4	10.7	30.0	0.98	3.1	11
Grapefruit juice (sweetened)	58.4	15.7	5	9.1	40.0	1.90	3.1	13

<sup>1</sup> Microscopic evaluation.

instance was the average weight of the enamel of the upper molars of the test animals less than that of the controls.

The values in the table, including the microscopic evaluation, are averages of all 6 lower molars. The degree of erosion was not always the same in the different teeth. In some instances it was pronounced in 2 or 3 teeth but slight in others; or, in some cases there was slight erosion in several of the teeth and none in the remainder. Averaging the degree of erosion of a number of teeth with such variations would ac-



cordingly give fractional values. The fractions, however, are omitted in the table because the nearest whole number is sufficient to indicate whether the extent of erosion was the same or different in the teeth of the rats on the various juices.

The juices are arranged serially in the table in accordance with the increasing loss in weight of the enamel of the lower teeth of the test animals. The loss in weight of the entire tooth and of the enamel was determined by taking the control values as the norms. In the case of the entire tooth it may not have been due in every instance entirely to the loss of the enamel for in some teeth in which the degree of microscopic erosion averaged 3 to 5, the erosion was so deep in places that it seemed to the observer that there had been some destruction of the dentine also.

The erosion produced by tomato and prune juice, as seen under the microscope, was relatively slight whereas with the other juices it was definitely pronounced. As will be observed from a comparison of the data in the table there was good correspondence between the loss in weight of the enamel and the subjective evaluation of the degree of erosion, except in 1 instance. The microscopic erosion produced by pineapple juice was less than that caused by orange juice, whereas the loss in weight of the enamel was slightly greater on the pineapple juice.

The pH of each bottle or can of the juices used in the experiment was determined with the glass electrode. The variation from sample to sample did not exceed 0.3 pH; in most instances, it was within 0.1 pH. Titratable acidity was determined electrometrically by titrating to the pH end-point of the predominating acid.

There seemed to be a general tendency for the degree of erosion to increase with an increase in the acidity of the juice ingested as expressed in terms of either hydrogen ion concentration or per cent titratable acidity. However, it should be noted that there was not a strict and invariable correspondence between the acidity and the amount of tooth destruction. Apple juice, for example, was less acidic than pineapple juice

but produced a much greater amount of erosion and loss of enamel. There was considerable difference in weight loss of enamel caused by prune and orange juice, although the pH of the 2 juices was the same. Similar results were obtained with grape and grapefruit juice.

*Part 2. Fruit juices constituting a portion of the  
total fluid intake*

From the standpoint of dietetics, the criticism may be raised against the preceding experiments that in the human consumption of the fruit juices, it is customary to drink them once or at most several times a day. Consideration of this fact suggested the advisability of the following study.

The procedure was the same as in Part 1 except that the test animals were given tomato, orange or grapefruit juice 3 times a day and when this had been consumed were then allowed to drink water *ad libitum*. As in the preceding experiments sugar was added to the grapefruit juice to make it palatable.

Each day except Sundays the test animals were given the juices in an amount equal to one-third the water intake of the preceding day. This was divided into 3 equal portions which were presented to the animals in graduated drinking tubes early in the morning, at noon, and again in the late afternoon. Usually the juices were consumed promptly. This procedure was continued for 200 days, at the end of which time the animals were sacrificed and the teeth removed for study as in the preceding experiment. In the course of the experiment each test animal consumed approximately 1.2 liters of fruit juice which was equivalent to 23% of its total water intake. The total intake of the juices was less than one-third of that of water because water alone was drunk on Sundays.

A comparison of the results presented in table 2 with those in table 1 shows that there was a loss in the weight of the enamel of the test animals on the various juices, but that it was considerably less than when these juices served as the

sole source of fluid. The loss in weight of the entire tooth was extremely small and was not of statistical significance. This was doubtless because the enamel constitutes such a small portion of the entire tooth. A 10% destruction of the enamel would amount to only about 2% of the weight of the entire tooth.

TABLE 2

*Effects of various juices on the lower molar teeth of the white rat when ingested 3 times a day for 200 days.*

FLUID CONSUMED	WEIGHT OF LOWER MOLARS	LOSS IN WEIGHT	DEGREE OF EROSION	WEIGHT OF ENAMEL	LOSS IN WEIGHT OF ENAMEL	NUMBER OF ANIMALS
	mg	%		mg	%	
Water	69.0	..	0	12.6	..	12
Tomato juice	68.2	1.2	0	11.8	6.3	12
Orange juice	67.0	2.9	1	11.0	12.7	12
Grapefruit juice (sweetened)	67.4	2.3	2	10.1	20.0	12

#### DISCUSSION

The lack of a strict correspondence between the extent of erosion and the degree of acidity of the ingested fluid observed in these experiments would appear to confirm the conclusions of McClure and Ruzicka ('46) that there are other factors besides acidity which produce a decalcification of enamel and dentine *in vivo*. In their experiments the citrate ion had a marked local decalcifying action, whereas no such action was caused by the lactate ion, although the pH of the citrate and lactate drinking fluid was the same. In our experiments there were striking exceptions to what appeared to be a general tendency toward greater decalcification with increasing acidity. Other investigations (Gortner, McCay, Restarski and Schlack, '46) have shown that oxalic acid with a pH as low as 2.1 had no etching action on the enamel of the teeth of rats, but produced a hard deposit which afforded protection against decalcification by phosphoric and citric acid.

Further investigations of the decalcifying action of different ions, as suggested by the work of McClure and Ruzicka

('46) should yield profitable results. Since our experiments were not undertaken with this purpose in mind, there was not sufficient control of certain variables to permit definite conclusions with regard to the action of specific ions. However, it is of interest to re-examine our data in the light of the compilation of the acid constituents of fruits (Hartmann and Hillig, '34) given in table 3.

Juices from the 3 fruits containing the highest concentration of citric acid produced marked erosion. Grapefruit juice, which contains more citric than orange and pineapple juice, caused much greater erosion. However, grape juice which contains practically no citric acid and relatively little malic,

TABLE 3

*Acid constituents of various fruit juices (Hartmann and Hillig, '34).*

	CITRIC	MALIC	TARTARIC
	%	%	%
Orange, Florida	0.92	0.18	...
Grapefruit	1.33	0.08	.
Grape, Concord	0.02	0.31	1.07
Tomato	0.47	0.05	.
Prune (Italian style)	none	1.44	..
Apple	0.0-0.03	0.27-1.03	..
Pineapple	0.77	0.12	

but a large amount of tartaric acid caused erosion which was almost as prominent as that produced by grapefruit juice. The results obtained with prune and apple juice are difficult to explain. Neither contains citric acid. Prune juice is much richer in malic acid and yet it produced considerably less erosion than apple juice. Apparently we must look for some other factor than malic acid alone to account for the erosive action of apple juice.

In view of the fact that the erosion of teeth by acid-containing beverages is a topical and not a systemic effect, it was not considered necessary to record the amount of food consumed which, as stated previously, was eaten *ad libitum*. In this connection it should be noted (table 1) that the loss in

weight of the teeth paralleled the degree of erosion as determined by microscopic examination.

It is possible that the sugar in the grapefruit juice may have accentuated the erosive action of the acid in the juice (Gortner, Restarski and McCay, '45). The primary purpose of this investigation, however, was not to study the mechanism of erosion by acid-containing beverages, but to obtain comparative data on the erosive action of various juices which appear to have become fairly common articles of human consumption.

While these and other experiments of a similar nature that have been reported in the literature may ultimately contribute toward the solution of the enigmatic problem of erosion and caries in human teeth, in our opinion, it would be inadvisable to apply them as a guide for a dietary regimen without further extensive investigation. In the first place, the amount of fruit juices ingested by the animals, even in the experiments in Part 2, is relatively much greater than what may be assumed to be the average human consumption. Secondly, the drinking mechanism in the rat is different from that in man. The rat laps its fluid and it is not improbable that the fluid in passing through the mouth may be in contact with the teeth longer than in the human mouth. This is a matter which merits closer study.

#### SUMMARY AND CONCLUSIONS

Erosion in the lower molar teeth of the albino rat resulted from the daily ingestion of each of the following canned fruit juices when they served as the sole fluid intake over a period of 100 days: apple, grape, orange, tomato, sweetened grapefruit, pineapple and prune.

The greatest amount of erosion occurred in the teeth of the animals on apple, grape, and grapefruit juice, the least in those of the animals on tomato and prune juice.

Erosion of a milder degree also resulted from the ingestion of tomato, orange, and sweetened grapefruit juice 3 times a day for 200 days.

There appeared to be some relationship but not a strict correspondence between the degree of acidity of the juices and the amount of tooth destruction.

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## THE PHYSIOLOGICAL AVAILABILITY OF PANTOTHENYL ALCOHOL<sup>1, 2</sup>

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### ONE FIGURE

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Relatively few compounds related to pantothenic acid have been found to display vitamin activity. Nease ('43) has listed 26 derivatives and analogues of pantothenic acid which had been tested for pantothenic acid activity by microbiological methods. Only one, hydroxypantothenic acid, showed any significant activity, which varied from 1.5 to 25% of pantothenic acid, depending upon the organism used and the assay conditions (Mitchell et al., '40). Zschiesche and Mitchell ('40) demonstrated that hydroxypantothenic acid has growth-promoting properties for rats also, although of a lesser magnitude than pantothenic acid, which could not, however, be stated quantitatively because of the small number of rats used.

The most active analogue which has been described is pantothenyl alcohol:  $\alpha, \gamma$ -dihydroxy-N-(3-hydroxypropyl)- $\beta, \beta$ -dimethylbutyramide, the alcohol analogue of pantothenic acid. Pfaltz ('43) found that pantothenyl alcohol is approximately as effective as calcium pantothenate with regard to influence on growth and achromotrichia of black rats and alopecia in mice. Burlet ('44) demonstrated that pantothenyl alcohol is

<sup>1</sup> A preliminary report of this work was presented before the Division of Agricultural and Food Chemistry at the 111th meeting of the American Chemical Society, April 14, 1947, Atlantic City, New Jersey.

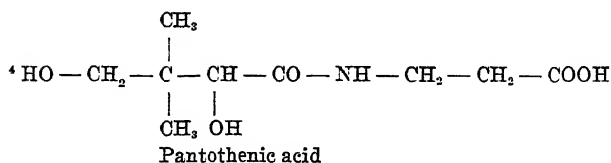
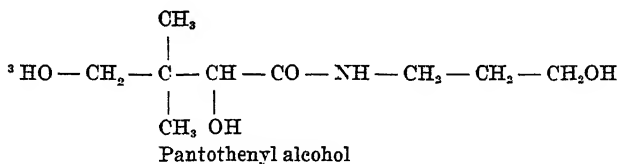
<sup>2</sup> Roche Publication no. 97.



converted by the rat and by man to pantothenic acid, as judged by urinary excretion of pantothenic acid. In another vein, Shive and Snell ('45) reported that pantothenyl alcohol is an inhibitor for several microorganisms at molar inhibition ratios of 700–200,000. The present study concerns further comparative investigation of the physiological availability of pantothenyl alcohol<sup>3</sup> and calcium pantothenate<sup>4</sup> to normal humans.

#### EXPERIMENTAL

The human availability technique used in these studies was an adaptation of the human bioassay method of Melnick et al. ('45). Seven healthy male adult subjects participated in these excretion experiments. Pantothenic acid in the urines was determined by the method of Atkin et al. ('44) employing *Saccharomyces carlsbergensis* 4228, and by the method of Skeggs and Wright ('44) employing *Lactobacillus arabinosus* 17-5. Pantothenyl alcohol or pantoyl lactone at levels of 0.05 to 0.5 µg per tube, with or without equal amounts of pantothenic acid, did not stimulate or inhibit either of these 2 organisms. Extensive assays of basal urines of all subjects prior to actual dosage yielded an average value of 3.7 mg per day (range 2.1–5.0). These values are of the same order as those reported by previous workers (Sarett, '45). The diets were not controlled rigorously although each subject avoided foods high in pantothenic acid and vitamin supplements. Twenty-four hour samples of urine were collected in amber jars containing 10 ml of toluene, the first voiding of the first



day being discarded. Equivalent doses of d(+)-pantothenyl alcohol or calcium d(+)-pantothenate, dissolved in distilled water, were taken orally immediately after the midday meal and the urine collections continued for either 1, 3 or 5 consecutive days. The dose-response relationship was established by feeding various levels of calcium pantothenate and measuring the extra urinary pantothenic acid excretion; this relationship is illustrated graphically in figure 1.

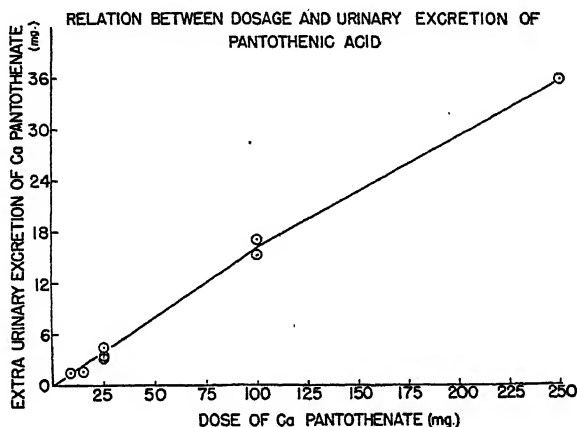


Fig. 1 Relation between dosage and urinary excretion of pantothenic acid.

Burlet ('44) demonstrated a greater excretion of pantothenic acid (ca. 50–100%) in the urines of 2 young men given 250 mg of pantothenyl alcohol as compared with 250 mg of calcium pantothenate. In this laboratory, 100 mg of calcium pantothenate (equivalent to 0.42 mM of pantothenic acid) was chosen as the first test dose and the urines were collected for a 24-hour period. This dose provides an excretion which is on the linear portion of the dose-response curve and which is large enough to minimize any errors due to possible fluctuations in the basal excretions. One week later, an equivalent amount of pantothenyl alcohol (86 mg) was given to the same subjects and the urines were collected for 24 hours. A summary of the data is presented in table 1, from which it is apparent (experiment I) that a decidedly greater excretion

TABLE 1

*Summary of human availability studies at dosage levels of 0.42 mM and 1.05 mM taken after lunch.*

EXPT. NO.	SUBSTANCE TAKEN	ASSAY ORGANISM	EXTRA EXCRETION OF PANTOTHENIC ACID IN URINE IN MG/DAY ON DAY					TOTAL EXTRA EXCRETION IN MG
			1	2	3	4	5	
I	100 mg calcium pantothenate	S. Carlsbergensis	11.5					11.5
		L. Arabinosus	11.3					11.3
	86 mg pantothenyl alcohol	S. Carlsbergensis	18.8					18.8
		L. Arabinosus	21.3					21.3
	Availability of pantothenyl alcohol = $\frac{20.1}{11.4} \times 100 = 176 \pm 28\%$ <sup>1</sup>							
II	86 mg pantothenyl alcohol	S. Carlsbergensis	19.3	3.9	3.3			26.5
		L. Arabinosus	21.7	4.4	3.0			29.1
	100 mg calcium pantothenate	S. Carlsbergensis	17.8	5.1	2.5			25.4
		L. Arabinosus	19.2	5.0	2.3			26.5
	Availability of pantothenyl alcohol = $\frac{27.8}{26.0} \times 100 = 107 \pm 6.9\%$ <sup>1</sup>							
III	86 mg pantothenyl alcohol	S. Carlsbergensis	18.9	2.3	2.0	1.0	1.0	25.2
		L. Arabinosus	18.7	1.6	1.2	0.4	0.4	22.3
	86 mg pantothenyl alcohol	S. Carlsbergensis	19.5	3.2	1.9	0.7	0.6	25.9
		L. Arabinosus	18.5	2.7	2.2	1.8	1.4	26.6
	100 mg calcium pantothenate	S. Carlsbergensis	17.0	2.3	0.9	0.4	0.8	21.4
		L. Arabinosus	16.9	2.1	0.4	0.4	1.4	21.2
	Availability of pantothenyl alcohol = $\frac{25.0}{21.3} \times 100 = 117 \pm 8.5\%$ <sup>1</sup>							
IV	250 mg calcium pantothenate	S. Carlsbergensis	38.8					38.8
		L. Arabinosus	44.3					44.3
	215 mg pantothenyl alcohol	S. Carlsbergensis	61.1					61.1
		L. Arabinosus	66.4					66.4
	215 mg pantothenyl alcohol	S. Carlsbergensis	61.4					61.4
		L. Arabinosus	68.0					68.0
	250 mg calcium pantothenate	S. Carlsbergensis	32.4					32.4
		L. Arabinosus	34.5					34.5
	253 mg sodium pantothenate	S. Carlsbergensis	33.7					33.7
		L. Arabinosus	39.9					39.9
	Availability of pantothenyl alcohol = $\frac{64.2}{37.5} \times 100 = 171 \pm 14.7\%$ <sup>1</sup>							
	Availability of sodium pantothenate = $\frac{36.8}{37.5} \times 100 = 98 \pm 13.3\%$ <sup>1</sup>							

<sup>1</sup> Standard error of the quotient.

of pantothenic acid occurred after ingestion of pantothenyl alcohol than after the ingestion of an equivalent amount of calcium pantothenate. These results apparently were in good agreement with those of Burlet ('44).

In order to test whether the greater excretion of pantothenic acid after the ingestion of pantothenyl alcohol might be due to more rapid excretion, the experiment was repeated with the urine collection period extended to 3 consecutive days. The results are given in table 1 (experiment II). In this experiment, contrary to the first one, the large differences between the pantothenic acid excretions disappeared after the ingestion of equivalent doses of calcium pantothenate and pantothenyl alcohol. This is true of the first-day excretion as well as of the total 3-day excretion. It is therefore probable that a differential rate phenomenon is not involved, but that the degree of saturation of the subjects is important. The first test dose (calcium pantothenate) shows a markedly lower level of excretion than the second, third and fourth test doses (pantothenyl alcohol and calcium pantothenate), and since the latter are comparable to each other, a priming dose of about 100 mg is indicated.

With this in mind, the experiment was repeated with urine collections continued for 5 consecutive days. Two trials with pantothenyl alcohol were made 1 week apart and then 1 trial with calcium pantothenate. Determinations of basal values during and after this experiment revealed a slight increase in basal pantothenic acid excretion; these higher figures were used in computing the extra urinary excretion. Since the 2 trials with pantothenyl alcohol agreed very well, the results were averaged and then compared with the excretion after the dose of calcium pantothenate was given. The results are shown in table 1, wherein it can be seen that the results of the second and third experiments are in good agreement and indicate a slight superiority of pantothenyl alcohol over calcium pantothenate at the level fed.

It is apparent from the results of the second and third experiments in table 1 that the physiological availability of

pantothenyl alcohol lies between 107 and 117% of that of calcium pantothenate under the stated conditions. The first experiment is not included in this calculation, since, as stated above, the subjects were apparently not saturated with respect to calcium pantothenate.

Since Burlet ('44) had found a greater excretion of pantothenic acid after ingestion of a 250 mg dose of pantothenyl alcohol than after an equal weight of calcium pantothenate was given, the excretion experiments were extended with doses of 250 mg of calcium pantothenate and equivalent amounts of pantothenyl alcohol (215 mg) taken alternately 1 week apart. Basal urines were collected on the first day, the dose given on the next day, and the urines again collected during the next 24 hours. The results (table 1, experiment IV) show, in agreement with Burlet, that considerably more pantothenic acid is excreted after dosages of pantothenyl alcohol at this level.

The role of the cation was studied in an experiment with an equivalent dose of sodium pantothenate. From table 1 it is evident that the relative availability of sodium pantothenate is of the same order as that of calcium pantothenate, and the relative availabilities of both are distinctly lower than that of pantothenyl alcohol at this dose of 1.05 mM. Therefore the presence of calcium in the molecule is probably not a factor in the lower excretion as compared to pantothenyl alcohol when the test doses are taken after lunch, as was the case in the preceding experiments.

Silber ('45) has demonstrated that the presence of food in the gastrointestinal tract diminishes the absorption of calcium pantothenate in dogs fed amounts comparable to those in this experiment, namely 4 mg per kilo. As the doses in the present experiments were given directly after the midday meal, it was thought that this factor might be operative. Trials were therefore undertaken in which the doses were given on an empty stomach. The subjects refrained from eating breakfast on the morning of the experiment, took either 215 mg of pantothenyl alcohol or 250 mg of calcium pantothenate at 9 A.M., and

continued fasting until 12:30 P.M. Urines were collected for 24 hours. Complete excretion data for the first experiment and a summary of data for a second experiment are shown in table 2. The average urinary excretion of pantothenic acid by all subjects is greater after ingestion of the pantothenyl alcohol than after calcium pantothenate was given under these conditions. However, the excretions of 2 subjects, E.D.R. and S.H.R., are distinctly lower than those of the others after ingestion of calcium pantothenate but are within the general range after pantothenyl alcohol was administered. If the excretion values are averaged without these subjects, the differences between the excretions largely disappear after doses of the 2 compounds. The experiment was repeated with the dose of calcium pantothenate given first and again these 2 subjects excreted lesser amounts of pantothenic acid after taking calcium pantothenate. A summary of the relative availability values for pantothenyl alcohol is given in table 2. The average availability in experiments I and II, including all subjects, is  $130 \pm 31\%$ . However, when the values for the excretion of the 2 subjects, E.D.R. and S.H.R., after doses of both pantothenyl alcohol and calcium pantothenate are excluded, the availability of pantothenyl alcohol is  $101 \pm 12\%$ . Apparently these 2 subjects utilized pantothenic acid less efficiently than pantothenyl alcohol when ingested in the postabsorptive state.

Further inspection of table 2 shows that the values for extra excretion of pantothenic acid obtained by assay with *L. arabinosus* are higher than those obtained with *S. carlsbergensis*. A statistical analysis of these differences is given in table 3. After the lesser dose of calcium pantothenate, the difference is not significant, but at the lower level of pantothenyl alcohol the difference is significant at the 5% level but not at the 1% level. At the higher levels of both calcium pantothenate and pantothenyl alcohol, the differences are highly significant even at the 1% level.

Since these differences were greater after the dosage with pantothenyl alcohol, it was thought that perhaps some panto-

TABLE 2

*One-day excretion of pantothenic acid after a dose of 215 mg pantothenyl alcohol or 250 mg calcium pantothenate taken in the postabsorptive state. All values are in milligrams.*

SUBJECT	DOSE: 215 MG PANTOTHENYL ALCOHOL						DOSE: 250 MG CALCIUM PANTOTHENATE					
	Assay with S. Carlsbergensis			Assay with L. Arabinosus			Assay with S. Carlsbergensis			Assay with L. Arabinosus		
	Excretion:			Excretion:			Excretion:			Excretion:		
	Basal	Total	Extra	Basal	Total	Extra	Basal	Total	Extra	Basal	Total	Extra
Experiment I												
E.D.R.	5.9	71.8	65.9	6.9	108.0	101.1	8.4	17.5	9.1	10.4	19.9	9.5
J.M.C.	9.1	69.5	60.4	11.8	86.3	74.5	10.7	71.5	60.8	13.3	76.7	63.4
E.H.	5.5	62.3	56.8	5.8	67.2	61.4	7.9	73.3	65.4	8.5	75.8	67.3
S.H.R.	3.1	80.6	77.5	4.4	89.9	85.5	4.6	47.9	43.3	5.3	46.2	40.9
J.S.	6.5	81.4	74.9	7.7	93.8	86.1	9.2	91.7	82.5	9.7	93.9	84.2
F.W.J.	6.5	94.5	88.0	7.3	120.0	112.7	9.1	83.5	74.4	11.2	88.9	77.7
Average	6.1	76.7	70.6	7.3	94.2	86.9	8.3	64.2	55.9	9.7	66.9	57.2
Average excluding E.D.R. and S.H.R.	6.9	76.9	70.0	8.1	91.8	83.7	9.2	80.0	70.8	10.7	83.8	73.1
Experiment II												
Average	8.0	74.1	66.1	9.1	82.7	73.6	7.0	65.2	58.2	7.2	64.5	57.3
Average excluding E.D.R. and S.H.R.	9.0	81.8	72.8	9.2	91.4	83.2	9.1	93.4	84.3	9.2	89.6	80.4
Per cent relative availability of pantothenyl alcohol <sup>1</sup>												
Organism		Experiment I		Experiment II		Mean						
All subjects	S. Carlsbergensis	126 ± 26		114 ± 33		120 ± 30						
	L. Arabinosus	152 ± 33		128 ± 33		140 ± 33						
	Mean	139 ± 30		121 ± 33		130 ± 31						
E.D.R. and S.H.R. ex- cluded	S. Carlsbergensis	99 ± 12		88 ± 11		94 ± 12						
	L. Arabinosus	114 ± 13		102 ± 11		108 ± 12						
	Mean	107 ± 13		95 ± 11		101 ± 12						

<sup>1</sup>  $\frac{\text{Excretion after pantothenyl alcohol}}{\text{Excretion after Ca pantothenate}} \times 100 \pm \text{standard error of the quotient.}$

thenyl alcohol as such or pantoyl lactone was being excreted into the urine and might be acting as a stimulator for the organisms. Accordingly, to 3 of the urines which showed the greatest variation in level of pantothenic acid by the 2 organisms, were added equivalent amounts of pantothenyl alcohol, and to a duplicate series one-half an equivalent amount of pantothenyl alcohol and one-half an equivalent

TABLE 3

*Estimates of significance of differences between urinary pantothenate assays by L. Arabinosus and S. Carlsbergensis.*

SUBSTANCE TAKEN	DOSE	NO. SAMPLES	AVERAGE PANTOTHENATE ASSAY			SIG- NIFI- CANCE OF DIF- FER- ENCE (t)	VALUE OF t FOR SIG- NIFICANCE <sup>1</sup>	
			L. Ara- binosus	S. Carls- bergensis	$\frac{L. Arabin.}{S. Carlsb.} \times 100$		5%	1%
	mg		mg	mg	%		level	level
Calcium panto- thenate	100	21	21.9	21.5	102	0.34	2.086	2.845
Calcium panto- thenate	250	17	63.7	59.8	107	6.89	2.120	2.921
Panto- thenyl alcohol	86	25	24.8	23.7	105	2.44	2.064	2.797
Panto- thenyl alcohol	215	24	82.0	72.5	113	6.28	2.069	2.807

<sup>1</sup> Snedecor, G. W., Statistical Methods, p. 58, Collegiate Press, Inc., Ames, Iowa, 1938.

amount of pantoyl lactone. The urines were then diluted and assayed both by *L. arabinosus* and *S. carlsbergensis*. In no case, however, was there any significant difference from the values obtained in the assay of the original urines.

Wood ('47) has described a method for the computation of microbiological assays which also tests the validity of the assay. In this method the response is plotted against the logarithm of the dose. The pantothenic acid content of the



3 urines mentioned above was computed by this method as well as by the single curve method with essentially similar results.

#### DISCUSSION

The results show that with increasing dosage of the 2 compounds, pantothenyl alcohol shows increasingly superior availability when taken together with food. The connotations attached to the term "availability" in these experiments are somewhat different than usual, in that we are not concerned with the utilization of the same compound in different media, but rather in the behavior of 2 related compounds in the same medium.

Several factors may be considered in connection with the greater availability of pantothenyl alcohol. The evidence from the experiments in the postabsorptive state (table 2) and from Silber's ('45) experiments with dogs favors the view that, in the presence of food, calcium or sodium pantothenate is lost in the feces more readily than pantothenyl alcohol. Another possibility arises from the better stability of pantothenyl alcohol in acid solutions. It is well-known that pantothenic acid is readily hydrolyzed in dilute acid (Atkin et al., '44); experiments in this laboratory have shown that pantothenyl alcohol is considerably more stable than pantothenic acid in dilute acid, ca. pH 2 to 5 (unpublished data). It is therefore possible that greater destruction of the latter occurs in the stomach when the test doses are taken directly after lunch (table 1) than when taken in the postabsorptive state (table 2).

#### SUMMARY

The urinary excretion of pantothenic acid by 7 normal male subjects was slightly less after an oral dose of 100 mg calcium pantothenate than after an equivalent dose of pantothenyl alcohol, when taken after lunch. A priming dose of calcium pantothenate is necessary to insure valid results.

When the dose was increased to 250 mg of calcium pantothenate or 215 mg of pantothenyl alcohol, a decidedly greater excretion of pantothenic acid occurred after the ingestion of

pantothenyl alcohol. Similar results were obtained when sodium pantothenate was substituted for calcium pantothenate.

When the subjects received the greater dosages in the post-absorptive state, the differences in the pantothenic acid excretion disappeared largely except in the case of 2 of the 7 subjects.

The *L. arabinosus* assay values for pantothenic acid in the urines following the ingestion of the greater doses of both calcium pantothenate and pantothenyl alcohol were significantly higher than those obtained with *S. carlsbergensis*.

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# THE ENERGY EXPENDITURE FOR QUIET PLAY AND CYCLING OF BOYS SEVEN TO FOURTEEN YEARS OF AGE<sup>1</sup>

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ONE FIGURE

(Received for publication November 20, 1947)

Since the time of Lavoisier the energy requirement for different activities has been actively investigated but by far the greater part of the work has been carried out on adult subjects. The energy cost of many children's activities has not as yet been measured. This paper reports the results of measuring the energy expenditure of 7- to 14-year-old boys (a) sitting at quiet play and (b) cycling in the respiration chamber of the Nutrition Laboratory of Teachers College, Columbia University. Those reports found in the literature with which these results could be compared are cited under Results and Discussion.

## EXPERIMENTAL

Fifteen boys living in 2 homes for children in New York City served as subjects. Six of these boys served in successive years and were considered as new subjects each year, thus bringing the total number of cases up to 22. They were selected

<sup>1</sup> The data in this paper were taken from dissertations submitted by Clara M. Taylor ('37), Mina W. Lamb ('42) and Mary E. Robertson ('42) in partial fulfillment of the requirements for the degree of Doctor of Philosophy, under the Joint Committee on Graduate Instruction, Columbia University.

on the basis of age, height, weight, good health, and general spirit of cooperativeness displayed during preliminary tests. All of the boys were born in this country but varied in racial background. Three age groups were studied. Data for each group, with respect to age range, average weight, height, and deviation from predicted weight (Baldwin-Wood standards) are given in table 1.

TABLE 1

*Average weight, height, deviation from predicted weight, and basal metabolism of boys. (Figures in parentheses show range.)*

SUBJECTS	BODY WEIGHT		BODY HEIGHT		DEVIATION FROM PREDICTED WEIGHT <sup>1</sup>	BASAL METABOLISM IN CALORIES PER			
	lb	kg	inch	cm		24 hr.	kg/hr.	sq.m./hr.	cm of ht./24 hr.
Group I:									
6 subjects	47.6	21.6	46.4	117.9	-2.9	1030	2.0	51.7	8.8
with ages	(41.1-	(18.7-	(42.5-	(108.1-	(-5.2-	(881-	(1.7-	(43.9-	(7.9-
ranging	56.1)	25.5)	50.4)	127.9)	+2.8)	1087)	2.2)	54.5)	9.2)
6-6 to 8-4 <sup>2</sup>									
Group II:									
6 subjects	63.3	28.8	52.0	132.0	-1.3	1101	1.6	44.4	8.3
with ages	(50.6-	(23.0-	(49.4-	(125.5-	(-10.1-	(1009-	(1.4-	(41.2-	(7.8-
ranging	72.0)	32.7)	54.7)	138.8)	+4.3)	1309)	1.9)	48.2)	9.5)
9-5 to									
11-1 <sup>2</sup>									
Group III:									
10 subjects	90.3	41.0	58.2	149.1	+1.0	1277	1.3	40.6	8.5
with ages	(68.1-	(31.0-	(54.7-	(138.9-	(-6.9-	(1059-	(1.2-	(38.9-	(7.6-
ranging	117.8)	53.6)	64.4)	163.6)	+15.3)	1604)	1.5)	43.9)	9.8)
12-4 to									
14-1 <sup>2</sup>									

<sup>1</sup> Deviations from Baldwin-Wood standards.

<sup>2</sup> First number in each set refers to years, the second to months.

The basal metabolism was determined by means of the Sanborn-Benedict portable respiration apparatus, the Collins-Benedict-Roth respiration apparatus, or the Benedict and Benedict student respiration apparatus. Tests with the same

subject on these 3 forms of apparatus in this laboratory have repeatedly demonstrated that they may be used interchangeably for basal energy metabolism determinations. The determinations were made under the generally accepted standard conditions, i.e., in the period 12 to 18 hours after the last intake of food, lying still awake after a 30-minute rest period, body temperature normal and environmental temperature comfortable. Two 8- to 10-minute determinations were made each morning and the procedure repeated on other mornings until at least 3 of the lowest results were found to check within 5%. In most cases 2 mornings were sufficient to obtain acceptable results. The average results expressed in 4 ways are given in table 1. These averages have been used for comparison with the energy expenditure for quiet play and cycling.

The activity studies were carried out in an open circuit respiration chamber patterned after the respiration chambers used by Benedict et al. ('20) and Ritzman and Benedict ('29). The original chamber was described in Columbia University dissertations by Potgieter ('33), Robb ('34), and Williams ('34). After the completion of their studies, the chamber was moved and some alterations made in dimensions and lay-out. These new dimensions and lay-out were given by Taylor ('37). A diagram of the chamber with bicycle ergometer in place, the absorbing trains for carbon dioxide and the remote control board is shown in figure 1. The dimensions of the chamber are as follows: length, 222.3 cm; width, 171.0 cm; height, 226.8 cm; volume, 8,577 l.

At frequent intervals (usually once a week) the absorbing trains and the respiration chamber were tested for tightness by introducing a weighed amount of carbon dioxide directly into the chamber through the opening at "h" (fig. 1). A sample of the air in the chamber was taken before the carbon dioxide was introduced and again at the end of a 25-minute period of circulation through the absorbers to determine the percentage of carbon dioxide in the residual air of the chamber. The increase in grams of carbon dioxide in the

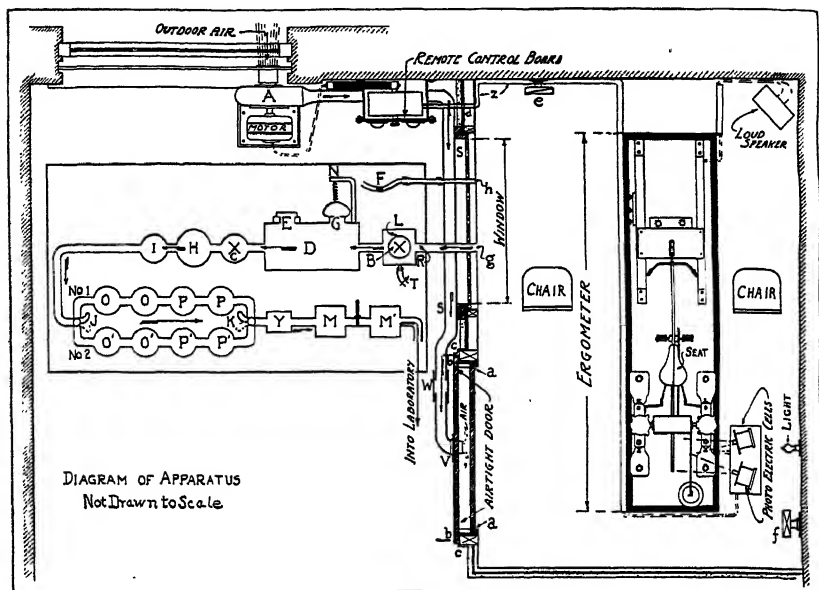


Fig.1 Diagram of Apparatus. A, blower for drawing in outdoor air and forcing it into chamber; B, blower for removing air from chamber and carrying it to wind chest; C, positive blower for forcing air from wind chest D through absorbing systems; E, metal cap closing wind chest; F, manometer for testing pressure of chamber air; G, rubber cap attached to spring for maintaining air pressure in chamber; H, large jar of sulfuric acid for drying chamber air; I, small jar of sulfuric acid, an added precaution for drying air; J, K, levers directing chamber air through absorbing train, I or II; L, tin box acting as housing for blower B; M, M', meters for measuring volume of air passing through chamber; N, support for spring and rubber cap; O, O and O', O', jars of soda lime for absorbing CO<sub>2</sub>; P, P and P', P', jars of H<sub>2</sub>SO<sub>4</sub> for absorbing moisture; R, metal tube connecting chamber with blower B; S, metal tubing for conducting outdoor air to chamber; T, small opening for introducing CO<sub>2</sub> for testing absorbing train; V, opening into door, permitting entrance of outdoor air; W, rubber tubing completing air passage into chamber; Y, metal receptacle containing cotton sprinkled with NaHCO<sub>3</sub> for neutralizing acid fumes in air leaving sulfuric acid jars; Z, electric cable carrying electric connections for magnetic core, photo-electric cell, red and green lights and loud speaker; a, a and b, b, felt connections on door frame; c, c, clamps for holding chamber door in place; d, opening in chamber wall for passage of electric cable; e, f, electric fans for evenly distributing chamber air; g, opening through which chamber air is drawn into absorbing system; h, opening for taking samples of chamber air.

chamber air plus the corrected grams of carbon dioxide absorbed by the absorbing system gives the total return on the weighed amount of carbon dioxide introduced. The average recovery of carbon dioxide throughout the investigations ranged between 99.3 and 99.9%.

The Carpenter ('23, '29, '33) modification of the Haldane gas analysis apparatus was used. This apparatus measures the carbon dioxide in air to the nearest 0.001%. Only those results agreeing within 0.004% were considered acceptable. The apparatus was tested for accuracy at frequent intervals by analyzing samples of outdoor air. Carpenter suggests that if a given sample of outdoor air is found to contain between 0.027 and 0.033% of carbon dioxide the apparatus may be considered to be in good working condition.

Plunger pumps, described by Lee ('33), were used for sampling the chamber air. The pumps were tested for tightness before each experiment. Usually the gas samples were analyzed within a few hours, although it was established that samples allowed to stand for as long as 24 hours showed no change in composition. These pumps have a capacity of 370 ml so that it is possible, if necessary, to make as many as 5 determinations on 1 sample.

For the cycling tests a bicycle ergometer<sup>2</sup> was used which was patterned after the bicycle ergometers of electric brake type designed by Benedict and Carpenter ('09), Benedict and Cady ('12) and Krogh ('13). Detailed description is given by Taylor ('37). In order that approximately the same amount of work should be done in each test period, a fairly constant speed of pedaling was maintained by placing a red and a green light on a support just below the handle bars where they could be easily seen by the cyclists. If the pedaling was too fast, the red light would show, and if too slow, the green light. The boys were instructed to pedal just fast enough to keep both lights out. This maintained a speed of 54 to 65 pedal revolu-

<sup>2</sup> The bicycle ergometer was built by personnel furnished by the Works Division of the Emergency Relief Bureau of New York City under the direction of Dr. Irving Lorge, Professor of Education, Teachers College, Columbia University.



tions per minute. This device interested the boys and resulted in steadier pedaling than when a metronome was used. The pedaling was done against the electric brake by passing a constant current of 0.6 ampere through the coils of the magnetic core between the pole faces of which the copper disc, which replaced the rear wheel of the bicycle, rotated (fig. 1). This was judged by experienced cyclists to simulate closely the work done against friction of tires and resistance of wind under ordinary conditions of cycling in open country.

In order to have some means of direct communication with the subjects in the chamber, a loud speaker was placed in 1 corner (fig. 1), and connected with a hand microphone which was hung on the control board.

#### PROCEDURE

The results reported in this paper are those of tests made in the afternoon after school,  $3\frac{1}{2}$  to 4 hours after a light to moderate lunch. At the beginning of the study it was established by Taylor ('37), by making tests on the same subjects, 17 in the morning before breakfast and 29 after school, that the agreement of results was so close as to justify the assumption of an average respiratory quotient of 0.82 in the afternoon after school. It was also established, as the result of 15 tests on each of the 3 age groups studied, that cycling at the rate of 49 to 66 pedal revolutions per minute did not cause oxygen debt. Hence, no recovery period was required and it was allowable to use the increase in carbon dioxide production during the cycling periods without making any correction for development of an oxygen debt or irregular production of carbon dioxide.

Two boys were studied at a time and treated as a single subject. Preceding each test, the boys were weighed and their heights determined. They were then given something to provide amusement for the quiet play period, the nature of this being limited to games, puzzles and other quiet activities, which would involve, for the most part, the use of only the

finger muscles with occasional arm movements. Also, they were told to keep their hands on the handle bars while cycling.

The chamber was thoroughly ventilated before each test and the ventilation continued for a 5-minute adjustment period after the boys had entered the chamber and the door had been closed. Then followed the quiet play period, lasting 15 minutes, at the end of which the signal to start pedaling was given. In groups II and III each boy pedaled for 15 minutes, making a total of 30 minutes of cycling. While 1 boy cycled, the other continued sitting at quiet play. In group I, it was found that a 15-minute cycling period was too long and consequently the 30-minute cycling period was divided so that each child in turn rode for 8 minutes and then for 7 minutes, making a total of 15 minutes each. During the 50 minutes when the boys were in the chamber, they were, on the whole, most cooperative.

Samples of chamber air were withdrawn by pumps at the beginning and end of each period and analyzed to obtain the increase in carbon dioxide content of the chamber air. These results were then corrected for the amount of carbon dioxide in the outdoor air circulated through the chamber during the tests. The absorbing trains (fig. 1) were weighed before the beginning and at the end of each period, one being used for the period of quiet play and the other for cycling. To obtain the total carbon dioxide produced, the increase in weight of each absorbing train, corrected for the amount of carbon dioxide of the outdoor air, was added to the corrected carbon dioxide of the chamber air. To obtain the average carbon dioxide produced per boy during the period of quiet play, the total carbon dioxide produced during the period was divided by 2. To obtain the average amount of carbon dioxide produced per boy cycling, the average carbon dioxide produced per boy sitting at quiet play for 30 minutes was deducted from the total carbon dioxide produced during the 30-minute cycling period.

## RESULTS AND DISCUSSION

The average results of 70 determinations of energy expenditure for quiet play and of 61 for cycling, including the cost of the basal metabolism, are given in table 2 along with the probable error of the mean, the coefficient of variation, and the probable error as percentage of the mean.

TABLE 2

*Average energy expenditure of boys for quiet play and cycling.  
(Including the basal metabolism.)*

	QUIET PLAY			CYCLING		
	Group I (7-8 yrs.)	Group II (9-11 yrs.)	Group III (12-14 yrs.)	Group I (7-8 yrs.)	Group II (9-11 yrs.)	Group III (12-14 yrs.)
CO <sub>2</sub> (gm) produced per child per hr.						
Mean $\pm$ P.E. <sup>1</sup>	23.06 $\pm 0.41$	25.24 $\pm 0.26$	28.63 $\pm 0.69$	48.77 $\pm 0.75$	50.59 $\pm 0.63$	61.37 $\pm 1.05$
No. of cases	19	29	22	19	20	22
C.V. <sup>2</sup> (%)	11.7	8.3	16.8	10.0	8.3	11.9
P.E. as % of mean	1.8	1.0	2.4	1.5	1.2	1.7
Cal. per kg per hr.						
Mean $\pm$ P.E.	3.1 $\pm 0.07$	2.6 $\pm 0.02$	2.1 $\pm 0.04$	6.6 $\pm 0.13$	5.1 $\pm 0.04$	4.5 $\pm 0.06$
No. of cases	19	29	22	19	20	22
C.V. (%)	14.2	6.5	13.3	12.3	5.7	9.3
P.E. as % of mean	2.3	0.8	1.9	2.0	0.8	1.3
Cal. per cm of ht. per hr.						
Mean $\pm$ P.E.	0.59 $\pm 0.01$	0.58 $\pm 0.01$	0.58 $\pm 0.01$	1.23 $\pm 0.02$	1.15 $\pm 0.01$	1.24 $\pm 0.02$
No. of cases	19	29	22	19	20	22
C.V. (%)	11.9	6.9	15.5	10.6	7.0	9.7
P.E. as % of mean	1.7	1.7	1.7	1.6	0.9	1.6

<sup>1</sup> Probable error.

<sup>2</sup> Coefficient of variation.

Considering the variability in the amount of involuntary movement of head, hands, and feet observed from day to day, coefficients of variation ranging from 5.7 to 16.8% are not great. That the coefficients of variation are in general lower

in the cycling period than in the period of quiet play may be due to activity being more closely controlled in the cycling period. The probable error of the mean expressed as per cent of the mean ranges from 0.8 to 2.4%, indicating the validity of the average results.

It will be noted that whereas energy expenditure for each activity in Calories per kilogram per hour decreases with the increase in age, there is practically no change when it is expressed in Calories per centimeter of height per hour.

Comparing the total cost of quiet play with the basal energy expenditure, the percentage increase for the 7- to 8-year-old boys is 55%, for the 9- to 11-year-old boys, 63%, and for the 12- to 14-year-old boys, 62%. For cycling, the increases above the basal metabolism are, respectively, 230, 219 and 246%.

There are few results in the literature with which to compare the findings of this study. For boys in the age range of group I Sondén and Tigerstedt (1895) reported an expenditure of 3.46 Cal. per kg per hr. for sitting quietly reading; Bedale ('23), for sitting quietly in the schoolroom, 2.40; for the quiet play of this study the average result was 3.13. For boys 9 to 11 years of age, sitting reading and eating apples Sondén and Tigerstedt reported 2.75; Olin ('16), for sitting quietly after breakfast, 1.81; Bedale, for sitting quietly in the schoolroom, 2.16; for the quiet play of this study the average result was 2.55. For 14- and 15-year-old boys sitting quietly, Bedale reported 1.96 and 1.90, respectively; for the quiet play of the 12- to 14-year-old group of this study the average result was 2.09. There are no reports in the literature on the energy expenditure of boys in cycling with which to compare the results of this study.

#### SUMMARY

Seventy determinations of energy expenditure for quiet play and 61 for cycling by 22 subjects, 7 to 14 years of age, are reported. A respiration chamber equipped with a bicycle ergometer of electric brake type was used. The results are

stated as the total expenditure, i.e., inclusive of the basal metabolism.

Nineteen determinations were made on boys 7 and 8 years of age. Sitting quietly at play the average energy expenditure of these boys was 3.1 Cal. per kg per hr., or 0.59 Cal. per cm of ht. per hr. For cycling, the average was 6.6 Cal. per kg per hr., or 1.23 Cal. per cm of ht. per hr.

Twenty-nine determinations of the energy expenditure in quiet play and 20 determinations for cycling were made on boys 9 to 11 years of age. For quiet play the average expenditure was 2.6 Cal. per kg per hr., or 0.58 Cal. per cm of ht. per hr.; for cycling, 5.1 Cal. per kg per hr., or 1.15 Cal. per cm of ht. per hr.

Twenty-two determinations were made on boys 12 to 14 years of age. For quiet play the average was 2.1 Cal. per kg per hr., or 0.58 Cal. per cm of ht. per hr.; for cycling, 4.5 Cal. per kg per hr., or 1.24 Cal. per cm of ht. per hr.

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## ERRATUM

Hale, E. B., C. W. Duncan and C. F. Huffman.

Rumen digestion studies. I. A method of investigating chemical changes in the rumen.

Journal of Nutrition, vol. 34, no. 6, December, 1947

Page 738: Line 4 from bottom, 1.8% to be changed to 3.6%

Page 741: The parentheses in formula 4 to be changed as follows:—

$$(4) \quad \frac{\text{Total amount of nutrient leaving rumen} - \left( \frac{\text{Amount of lignin leaving rumen}}{\text{Per cent lignin at 12 hours}} \times \frac{\text{Per cent nutrient in rumen at 12 hours}}{\text{Per cent nutrient in rumen at 12 hours}} \right)}{\text{Amount of nutrient in hay}} \times 100 = \text{Digestion coefficient of nutrient}$$

Page 743: Line 10 from top, the sentence starting with the words "In this instance rumen contents . . ." is to be changed to

"In this instance rumen contents are removed at the beginning of the digestion period (i.e., 12 hours after previous feeding) and again at the end of the digestion period under consideration."

## ERRATUM

Ershoff, Benjamin H.

The effects of B vitamins, liver and yeast on atabrine toxicity in the rat.

Journal of Nutrition, vol. 35, no. 2, February, 1948

Page 273—Table 3, Atabrine series C to be changed to

(9)						
C	10	41.8	99.1 ± 5.1	30.4 ± 2.7	303.8 ± 10.8	2.15





# THE RELATION OF DIET COMPOSITION AND VITAMIN C TO VITAMIN A DEFICIENCY<sup>1</sup>

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## ONE FIGURE

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Previous experiments conducted by the authors on the onset and development of the vitamin A deficiency syndrome in the rat resulted in 2 major observations.

The earliest observed effect of vitamin A deficiency in young growing animals is to decrease the efficiency of utilization of the food consumed, this being defined by the ratio "grams body weight gained per calorie ingested" (Mayer and Krehl, '48a).

Some of the earliest symptoms of vitamin A deficiency seen in growing rats are part of a syndrome that responds favorably to the administration of ascorbic acid (Mayer and Krehl, '48b), despite the fact that under normal conditions the rat can synthesize adequate amounts of this vitamin. This response to ascorbic acid, together with the fact that the symptoms observed such as hemorrhage of the lachrymal glands, bleeding of the gums, and swelling of the joints with occasional paralysis of the limbs are similar to those seen in the scorbutic guinea pig, supports the view that these symptoms

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exhibited by our vitamin A deficient rats are indeed scorbutic in nature.

An artificial, so-called "synthetic" diet was used in both studies, with all known vitamins (except vitamins A and C) added to the diet as crystalline supplements. The experiments reported in this paper constitute an extension of these early observations. The first part of this report deals with the determination of the influence of the nature and proportion of various components of the diet on the rate of growth, the survival time and the efficiency of food utilization. It was reasoned that if any significant protection is afforded by a given foodstuff, or any injurious effect is obtained by increasing the concentration of another foodstuff, these phenomena might be regarded as indicating some relation of that foodstuff to the normal metabolic role of vitamin A.

The second part of this report deals with further study of the interrelationship of vitamins A and C. An attempt at integration of all the results is presented in the discussion.

#### VITAMIN A DEFICIENCY AND DIET COMPOSITION

##### *Method*

It has been shown (Mayer and Krehl, '48a) that in the female rat the decrease in efficiency of food utilization for growth as the course of vitamin A deficiency progresses, deviates less markedly from that of the controls and is more delayed in its appearance than is the case with the male. Because of this and other known effects of sex on the course of vitamin A deficiency in the rat, the first study of the influence of diet composition on vitamin A deficiency was restricted to 1 sex, and females were chosen arbitrarily. (For purposes of the growth assay for vitamin A, however, the male rat is considered preferable to the female.) Weanling female rats of the Sprague-Dawley strain were used. To insure a low vitamin A reserve in the experimental animals, the mothers received a diet low in vitamin A during the latter half of the nursing period. The weanlings were placed on experiment

when 24 days old. Seventy-two animals were used, 12 on each diet, with 8 deficient and 4 control animals in each one of the 6 groups. Each of the diets shown in table 1 represents a group.

The animals were kept in individual screen-bottomed cages at constant temperature and humidity. They were weighed every 4 days. All diets and water were fed *ad libitum*. The experiment was continued until all deficient animals had died.

TABLE 1  
*Composition of diets used.*

INGREDIENTS OF BASAL MIXTURE <sup>1</sup>	DIET					
	A <sub>1</sub>	A <sub>2</sub>	A <sub>3</sub>	A <sub>4</sub>	A <sub>5</sub>	A <sub>6</sub>
	%	%	%	%	%	%
Casein <sup>2</sup>	25(25) <sup>3</sup>	10(10)	60(60)	39.4(25)	39.4(25)	25(25)
Corn oil	5(11)	5(11)	5(11)	7.9(11)	53.9(75)	5(11)
Sucrose	65.7(64)	80.7(79)	30.7(29)	.....	.....	.....
Dextrin	.....	.....	.....	.....	.....	65.7(64)
Lard	.....	.....	.....	46(64)	.....	.....
Salt mixture	4	4	4	6.3	6.3	4
Cystine	0.2	0.2	0.2	0.25	0.25	0.2
Choline	0.1	0.1	0.1	0.15	0.15	0.1

<sup>1</sup> Each of these basal mixtures was supplemented with the following vitamins in mg/kg (or mg/4,000 Calories for the high fat diets A<sub>4</sub> and A<sub>5</sub>):  $\alpha$ -tocopherol 100; 2-methyl-1,4-naphthoquinone 5; D<sub>2</sub> (viosterol) dissolved in corn oil 0.1; thiamine 5; pyridoxine 5; riboflavin 5; nicotinic acid 50; pantothenic acid 50; biotin 0.5; folic acid 0.5; inositol 100; para-aminobenzoic acid 100. Control rats received orally 1,200 I.U. vitamin A every 8 days.

<sup>2</sup> Vitamin-free "Labco."

<sup>3</sup> Figures in parentheses indicate the caloric distribution in per cent.

All diets used were of the "synthetic" type, being made up with purified ingredients and supplements of crystalline vitamins. The following major ingredients were used: Sucrose, dextrin, vitamin-free casein, refined corn oil, purified lard, and mineral salts mixture (Phillips and Hart, '35, modified by the addition of 0.02% CoCl<sub>2</sub>). Detailed information on the exact composition of the diets used including vitamin supplements is given in table 1.

Diets A<sub>1</sub>, A<sub>2</sub> and A<sub>3</sub> differed by the isocaloric replacement of sucrose with casein. A<sub>6</sub> was derived from A<sub>1</sub> by the replacement gram for gram of sucrose by dextrin to study the possible role of intestinal synthesis on vitamin A or its precursors. In diets A<sub>4</sub> and A<sub>5</sub> sucrose was replaced isocalorically by lard and corn oil, respectively, selected as examples of saturated and unsaturated fats.

### Results

Data on the effect of diet composition on the survival time and the maximum weight attained by the deficient animals,

TABLE 2  
*Survival time and maximum body weight as affected by the composition of the diet.*

RATION USED	VITAMIN A ABSENT		CONTROL
	Average survival time	Average maximum body weight	Body weight after 40 days
	<i>days</i>	<i>gm</i>	<i>gm</i>
A <sub>2</sub> (casein 10%, sucrose 79%) <sup>1</sup>	47 ± 4 <sup>2</sup>	92 ± 9	166 ± 19
A <sub>1</sub> (casein 25%, sucrose 64%)	39 ± 3	109 ± 6	180 ± 19
A <sub>3</sub> (casein 60%, sucrose 29%)	30 ± 4	107 ± 6	158 ± 3
A <sub>4</sub> (casein 25%, lard 64%)	52 ± 9	145 ± 8	209 ± 18
A <sub>5</sub> (casein 25%, corn oil 75%)	45 ± 7	97 ± 10	182 ± 12
A <sub>6</sub> (casein 25%, dextrin 64%)	38 ± 2	119 ± 13	193 ± 18

<sup>1</sup> These percentages refer to distribution of calories.

<sup>2</sup> Average and standard deviation.

and, for reference, the weights of the control groups after 40 days on the experiment are presented in table 2.

Consideration of groups A<sub>2</sub> (10% casein), A<sub>1</sub> (25% casein), and A<sub>3</sub> (60% casein) makes it obvious that increasing the quantity of dietary protein decreases the survival time, even though the average maximum weights attained by the different groups are probably not significantly different. The use of dextrin instead of sucrose (group A<sub>6</sub>) resulted in no benefit. The best results with respect to survival time, delay in onset of visible symptoms of vitamin A deficiency, and

average maximum weight attained by the deficient animals were obtained by the diet in which there was an isocaloric replacement of sucrose with lard (group A<sub>4</sub>). The similar use of corn oil (group A<sub>5</sub>) also resulted in some prolongation of survival time, although the average maximum growth of the deficient animals was very significantly inferior to that of the group fed the lard diet (A<sub>4</sub>). This was true, although there probably was no significant difference between the weights of the control rats on the 2 diets.

It will be noted that all of these diets supported good growth in the control animals. Kidney function tests (creatinine and

TABLE 3

*Effect of vitamin A deficiency on the efficiency of food utilization.*

RATION USED	GRAM GAIN IN BODY WEIGHT			
	Per gm of protein eaten		Per calorie eaten	
	Deficient	Control	Deficient	Control
A <sub>2</sub> (casein 10%, sucrose 79%) <sup>1</sup>	1.67	2.35	0.04	0.06
A <sub>1</sub> (casein 25%, sucrose 64%)	1.35	1.59	0.08	0.10
A <sub>3</sub> (casein 60%, sucrose 29%)	0.42	0.65	0.06	0.10
A <sub>4</sub> (casein 25%, lard 64%)	2.7	2.8	0.10	0.10
A <sub>5</sub> (casein 25%, corn oil 75%)	2.0	2.5	0.08	0.10

<sup>1</sup> These percentages refer to distribution of calories.

para-aminohippuric acid clearances) were conducted on the control rats, all of which were found to be normal.

Data on the effect of vitamin A deficiency on the efficiency of utilization of the food ingested are given in table 3, expressed both in "grams of body weight gained per gram of protein intake" and in "grams of body weight gained per calorie ingested" after 20 days on the experimental diet. It will be noted, by comparing groups A<sub>2</sub>, A<sub>1</sub> and A<sub>3</sub>, that the efficiency of utilization of protein ingested by the control animals decreases when the proportion of casein in the diet increases. On the lowest level of dietary protein (group A<sub>2</sub>), where the efficiency of utilization of dietary protein is high, the efficiency of food utilization is, however, lowest, appar-

ently because the animals increase their food consumption in order to ingest a sufficient amount of protein. If the protein level is sufficient to insure optimal growth (the animals on the low protein level are slow to start), the efficiency of food utilization in "grams body weight gained per calorie ingested" is the same on the various diets, in accordance with previous observations that specific dynamic action is not an important phenomenon in the growing animal.

A high protein intake makes more acute the decrease in efficiency of food utilization of the deficient animals compared to that of their controls. For example (table 3, diet A<sub>3</sub>, casein 60%), the grams gained per calorie consumed were 0.06 for the deficient animals compared with 0.10 for their controls, a decrease of 40%. Similarly, for the medium level of protein (diet A<sub>1</sub>) the drop was 20%. Unsaturated fat (corn oil, diet A<sub>5</sub>) compares unfavorably with saturated fat (lard, diet A<sub>4</sub>) in this respect. This point is considered in greater detail in the discussion.

The animals on the high protein, vitamin A deficient diet (A<sub>3</sub>) died exhibiting very marked symptoms which are considered as being scorbutic in nature (see introduction for interpretation). On autopsy, evidence of extensive abdominal hemorrhage was observed.

One of the chief and uniformly characteristic autopsy findings in animals which died as a result of vitamin A deficiency (especially with added vitamin C) was a greatly bloated and distended gastrointestinal tract. For example, the volume of the distended stomach alone of 1 rat was 25 ml for a 100 gm animal. A clue to the cause of this distension was given in 1 case in which autopsy immediately after death revealed that the esophagus of the animal had completely separated from the stomach, which had become sealed off at the cardia. This extremely bloated condition of the intestinal tract can be seen in figure 1. This abnormal intestinal tract helps explain (1) why the vitamin A deficient animal stops eating completely several days prior to death, and (2) why autopsy findings reveal an almost complete depletion of depot fat

reserves. A histological examination of sections made from the esophagus and the stomach at the cardiac sphincter and of the duodenum revealed the following: (a) the lumen of the esophagus was completely occluded with cellular debris consisting mainly of keratinized epithelium—this occlusion



Fig.1 Abdominal organs of a vitamin A deficient rat as seen at autopsy (dietary vitamin C added).

might have been the primary factor in causing the cleavage between the esophagus and the stomach; and (b) keratinization of the epithelium of the gastric and duodenal mucosa was evident. This was particularly marked in the duodenum, and in addition the height of the villi appeared to be less than



normal. A more complete pathological examination is being made and will be reported elsewhere.

In addition to these gastrointestinal symptoms which became evident only in the last stages of the deficiency prior to death, all the vitamin A deficient animals showed a deposition of dark red material around the nose and on the whiskers which was probably porphyrin in nature, as indicated by a characteristic fluorescence in ultra-violet light. As mentioned above, abdominal hemorrhage was often seen at autopsy but appeared less frequent and less severe in animals on the high fat rations.

Blood studies which were conducted on all deficient groups revealed a normal blood picture except in the high protein ( $A_3$ ) group. In this group hemoconcentration (hematocrit, cell volume 60%) and a low white blood cell count (average 5,000 instead of 15,000 for the other groups) were observed.

In most female animals the decline in weight was not continuous, but showed first a decrease, then a period of temporary increase, and then a second fall in weight terminated by death. The period of temporary increase lasted about 4 to 6 days and corresponded in all cases with the onset of puberty, evidenced by opening of the vagina. This regain in weight varied between little more than a plateau in the weight curve to an increase of about 40 gm. If the weight of the animals was still increasing at the onset of puberty, seen particularly with the high lard diet ( $A_4$ ), no such effect could, of course, be detected.

#### INTERRELATIONSHIP OF VITAMIN A DEFICIENCY AND VITAMIN C

##### *Method*

In the experiments dealing with the relationship between vitamins A and C, male rats of the Yale strain obtained from the Connecticut Agricultural Experiment Station were used first. They were weaned at 21 days and then placed on the experimental diet. The ration used was  $A_1$  (table 1). When ascorbic acid was added, the proportion used was 100 mg per

100 gm of diet. The Roe-Kuether ('43) method was used for the determination of ascorbic acid.

### Results

Twenty animals were used for blood and tissue determinations of ascorbic acid. They were given diet A<sub>1</sub> without vitamin C supplement. Control animals were given 1,200 I.U. of vitamin A every 10 days. Table 4 gives ascorbic acid values for the blood, adrenals and liver of these animals, both the deficient and the controls, after 37 days on the experimental regime. It will be seen that in all cases the values for blood and liver ascorbic acid of the vitamin A deficient animals are less than half of the normal. The adrenal ascorbic acid value is also very low.

TABLE 4

*Ascorbic acid content of adrenals, liver and blood of vitamin A-deficient male rats and their controls.<sup>1</sup>*

ANIMALS	ADRENALS	LIVER	WHOLE BLOOD
	mg/100 gm	mg/100 gm	μg/ml
Controls	355 ± 6(4)	34.5 ± 0.2(2)	16.1 ± 0.1(4)
Deficient in vitamin A	212 ± 35(11)	15.6 ± 1(3)	7 ± 1.5(11)

<sup>1</sup> Averages and standard deviations. Figures in parentheses indicate the number of animals studied.

It was also found, as might be expected in the few tests that it was possible to make, that the administration of vitamin C restored the adrenal, liver and blood ascorbic acid values to normal. Although the administration of vitamin A restored liver ascorbic acid to normal (28 mg per 100 gm), and had effected an increase in the adrenal ascorbic acid (average of 275 mg per 100 gm), the blood vitamin C level was still low (8 μg per ml).

In a second experiment, designed to test the effect of an addition of vitamin C to a vitamin A deficient diet on the development of vitamin A deficiency, 31 animals were used. The

animals were distributed in the following way (table 5): Four rats received vitamin A but no vitamin C, 10 received neither, 10 received both, and 7 received vitamin C but no vitamin A. The data in table 5 show that, although the control animals receiving vitamin A exhibited similar weight curves, the deficient animals behaved in a very different manner according to whether they were or were not provided with vitamin C

TABLE 5

*Survival times and body weights of vitamin A-deficient rats and their controls with and without vitamin C in the diet.*

ANIMALS	TIME ON EXPERIMENTAL DIET IN DAYS				
	20	30	35	40	80
Vitamin A-deficient rats					
Without vitamin C (10 animals)					
Body weight (gm)	159	137	175	...	...
Survival (%)	100	100	20	0	0
With vitamin C (7 animals)					
Body weight (gm)	148	212	229	245	302
Survival (%)	100	100	100	100	100
Control rats — received vitamin A					
Without vitamin C (4 animals)					
Body weight (gm)	225	303	343 <sup>1</sup>	373 <sup>1</sup>	...
Survival (%)	100	100	100	100	...
With vitamin C (10 animals)					
Body weight (gm)	214	304	328	354	505
Survival (%)	100	100	100	100	100

<sup>1</sup> The smallest of the original 4 control animals was sacrificed for analytical purposes. The survival and body weight data are therefore based on the remaining 3 larger animals.

in their diet. After 35 days on the experimental rations, all but 20% of the animals not receiving ascorbic acid had died; after 40 days all were dead. On the other hand, all of the vitamin A deficient animals which received vitamin C were still alive after 80 days. The maximum average body weight reached by the A-deficient animals not receiving ascorbic acid was 159 gm. The average weight of the vitamin A deficient

animals at 80 days was 302 gm, at which time the average weight of the controls was 505 gm.

That the effect of ascorbic acid in moderating the severe symptoms of vitamin A deficiency is not restricted to rats of Yale strain became evident when a group of 25 weanling male rats of the Sprague-Dawley strain (ordered specifically for purposes of vitamin A assay so as to have low vitamin A reserves) were placed on the vitamin A deficient diet ( $A_1$ ): 80% of the animals have survived for 80 days and attained an average weight of 245 gm. No xerophthalmia or marked visible evidence of vitamin A deficiency is apparent in these surviving animals. Of 6 control animals on the same diet but receiving vitamin A, all have survived, and their average weight at 80 days was 370 gm.

#### DISCUSSION

It must be noted that although many studies have been made on the role of various diet constituents on vitamin A absorption and utilization (Sherman, '40; Russell et al., '41; Ahmad, '31; Dutcher et al., '34; Muellder and Kelly, '42), no systematic investigation has been made on the influence of diet composition on the vitamin A deficiency syndrome. Furthermore, no studies (to our knowledge) have been conducted on vitamin A deficiency in the rat with a purely artificial (synthetic) diet: most workers, for example, have used up to 10% yeast as a source of B vitamins. Although it is difficult at present to ascribe a great significance to the use of synthetic diets beyond their ease of reproducibility and chemical purity, we believe that the influences of the level of dietary protein, of dietary fat, and of the replacement of saturated by unsaturated fat are especially significant in studies on vitamin A deficiency.

In the interpretation of the effect of protein level, however, a complication arises from the existence of an interrelationship between vitamins A and C. Although decreases in blood ascorbic acid in young bovines and urinary excretion of this vitamin in the rat have been associated with vitamin A de-

iciency (Boyer et al., '42), and similar relationships have been demonstrated for rat tissues (Sure et al., '39) and rat blood (Jonsson, Obel and Sjöberg, '42), the present and previous (Mayer and Krehl, '48b) experiments clearly point out that such findings are but mere reflections of the much more deep-seated pathological modifications that can be observed in the vitamin A deficient rat that does not receive added dietary or parenteral ascorbic acid.

As it has been demonstrated (Sealock and Silberstein, '39; Sealock, Perkinson and Basinski, '41, etc.) that ascorbic acid is needed for the metabolism of certain amino acids, phenylalanine and tyrosine in particular, it was theorized that perhaps the first underlying reason for the differences observed on the various protein levels is an increasing depletion of vitamin C caused by vitamin A deficiency, and that this is made more acute as the protein level is increased. This may explain why the deficient animals on the high protein diet in the first study in which vitamin C was not used died first and did so with very marked "scorbutic symptoms." It could also explain the fact that in the first experiment on the high protein diet the weight curves of the female control animals began to show a plateau when a weight of about 160 gm had been reached, after which there was a rapid gain in weight when ascorbic acid was added to the diet.

The experiment described in the first part of this paper on the influence of diet composition on vitamin A deficiency is now being repeated, but this time with vitamin C included in the diet. Preliminary results indicate that under these conditions the protein effect observed previously is in large measure cancelled. In fact, the effect of increasing the proportion of casein in the diet seems to be at least partly reversed, with the animals on the lowest level of protein showing the shortest survival time.

It seems logical to conclude, therefore, that the differences in survival time and efficiency of utilization on various levels of protein are not necessarily primary effects of vitamin A deficiency but secondary ones due to induced acute vitamin

C deficiency, which appears, as we have shown, many days, if not weeks, before acute symptoms of vitamin A deficiency are observed. On the other hand, the differences observed in the efficiency of food utilization between the animals fed a high saturated-fat diet (lard) and those given a high unsaturated-fat ration (corn oil) might well be a primary effect of vitamin A deficiency, inasmuch as certain authors (e.g., Monaghan and Schmidt, '32) have suggested that carotene and vitamin A may play a role in the oxidation of unsaturated fatty acids, and Sherman ('40) has indicated that there may be a metabolic interaction *in vivo* between vitamin A and linoleic acid.

The fact that an abnormally low white cell count was observed only in deficient rats fed a high level of protein suggests a possible interpretation of the controversial results obtained by various authors in their studies of the blood pictures of vitamin A deficiency in man. Hennessey ('32) and Sweet and K'ang ('35), for example, reported no effect of avitaminosis A on the white blood cell count, while Koessler, Maurer and Loughlin ('26) and Abbott and Ahmann ('38) found a decrease. We feel that possible differences in the composition of the diets used in these various studies might be responsible for this discrepancy.

The existence of a period of temporary recovery in body weight at the onset of puberty may be significant of the greater concentration in the blood of various hormones known to exist during that period. The possible influence of these hormones is now being investigated, together with the possibility that an increased rate of ascorbic acid synthesis may be involved.

The authors also feel it worthy of mention that a difference of efficiency of food utilization on the high saturated-fat diet was observed to develop more rapidly on a few animals placed in the cold (about 9°C.), which is known to cause an increased need for ascorbic acid (Therien and Dugal, '47).

It seems obvious that the existence of an interrelationship between vitamins A and C in the rat has an important bearing on any method of bioassay of vitamin A using this species.

## SUMMARY

Rats were maintained on vitamin A deficient diets whose compositions were varied with respect to protein, carbohydrate and fat. The following observations were made:

1. In vitamin A deficient animals, increased levels of dietary protein resulted in decreased efficiency of protein utilization for growth, decreased efficiency of food utilization, decreased survival time, and a general increased severity of the symptoms of vitamin A deficiency.

2. The isocaloric replacement of sucrose by fats afforded an increased protection against development of the syndrome of vitamin A deficiency. This was particularly noticeable with saturated fat (lard).

3. One of the first symptoms of the vitamin A deficiency syndrome was a depletion of the animal's vitamin C reserves, as evidenced by symptoms resembling scurvy and curable by ascorbic acid, as well as by decreases in the ascorbic acid content of liver, blood and adrenals. Evidence is presented suggesting that at least part of the effects of an increased protein level on vitamin A deficiency is mediated through the concomitant decrease in vitamin C reserve.

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# THE EFFECT OF ISOCALORIC SUBSTITUTION OF ALCOHOL FOR DIETARY CARBOHYDRATE UPON THE EXCRETION OF B VITAMINS IN MAN<sup>1</sup>

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The metabolism of ethyl alcohol by man supplies 7 calories per gram which can replace isodynamic quantities of carbohydrate and fat for energy (Atwater and Benedict, '02). There is evidence that ethyl alcohol is oxidized to acetaldehyde and that its further metabolism is coupled with pyruvic acid utilization (Leloir and Munoz, '38; Westerfeld, Stotz and Berg, '42, '43).

Clinical studies have associated "alcoholic" polyneuritis and pellagra with vitamin deficient diets (Shattuck, '28; Jolliffe, Colbert and Joffe, '36; Strauss, '35; Spies and DeWolf, '33). These reports did not show whether it was the lack of any specific vitamins required for the metabolism of the alcohol or the insufficiency of vitamins, protein and non-alcoholic calories which was responsible for the deficiency symptoms. Spies and DeWolf ('33) and Strauss ('35) were able to cure alcoholic pellagra and polyneuritis by feeding the subjects high caloric diets rich in good protein and B vitamin supplements, in addition to their daily whisky. In the report of

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Jolliffe, Colbert and Joffe ('36), it is seen that the caloric intake from non-alcoholic foods of most of the alcohol addicts with polyneuritis was grossly inadequate in contrast to that of the alcohol addicts without polyneuritis.

Lowry et al. ('42b) have shown a delayed development of polyneuropathy in rats on a thiamine deficient diet when alcohol was substituted isocalorically for part of the food intake, or when alcohol was added to the diet of pair-fed animals in lieu of water. Isocaloric replacement of alcohol for fat or for carbohydrate in the thiamine deficient ration of pigeons showed a similar sparing action of alcohol by its delay of onset of opisthotonus and death (Westerfeld and Doisy, '45).

In an effort to obtain further evidence concerning the relationship of alcohol metabolism in man to the requirements for thiamine and the other B vitamins, the present study was undertaken. The urinary excretion of several of the B vitamins was measured during experimental periods in which the only dietary change was the isocaloric substitution of ethyl alcohol for part of the carbohydrate (cornstarch and sugar) of the diet, with no significant alteration in the other dietary components.

#### EXPERIMENTAL

The subjects were 2 well-nourished adult males in good health (S and B, ages 31 and 44, weighing 75 and 82 kg, respectively). The composition of the experimental carbohydrate diet is shown in table 1. The daily menus were alternated as shown and were calculated to provide an adequate diet as summarized in table 1. In the given diet, the cornstarch, sugar and milk were served as puddings with the meals. This cornstarch and sugar supplied 1000 cal. per day and did not contain any appreciable amounts of vitamins, fat or protein. For the alcohol diet, four-fifths of the cornstarch and sugar were isocalorically replaced by 115 gm (800 cal.) of pure ethyl alcohol (150 ml of 95%). Water was added to give a volume of 10 oz. (approximate composition of whisky) and this was suitably diluted with water for consumption at the following

TABLE 1  
*Composition of carbohydrate diet.<sup>1</sup>*

MENUS FOR ALTERNATE DAYS			
Food	Amount	Food	Amount
	gm		gm
Breakfast			
Egg . . . . .	50	Egg . . . . .	50
Bacon . . . . .	20	Bacon . . . . .	20
Orange juice . . . . .	120	Orange juice . . . . .	120
Toast . . . . .	50	Toast . . . . .	50
Butter . . . . .	10	Butter . . . . .	10
Coffee . . . . .	180	Coffee . . . . .	180
Dinner			
Ground meat, cooked, wt. . . . .	100	Roast beef, cooked, wt. . . . .	100
Onions . . . . .	50	Lettuce . . . . .	30
Lettuce . . . . .	30	Tomatoes . . . . .	50
Asparagus, canned . . . . .	50	Mayonnaise . . . . .	14
Mayonnaise . . . . .	14	Green peas, canned . . . . .	100
Carrots, canned . . . . .	100	Bread . . . . .	50
Bread . . . . .	50	Oleo . . . . .	10
Oleo . . . . .	10	Cornstarch . . . . .	35
Cornstarch . . . . .	35	Sugar . . . . .	95
Sugar . . . . .	95	Milk . . . . .	240
Milk . . . . .	240		
Supper			
Mor beef, canned <sup>2</sup> . . . . .	100	Mor beef, canned . . . . .	100
Corn, yellow, canned . . . . .	100	Spinach, canned . . . . .	100
Potatoes, cooked, wt. . . . .	150	Potatoes, cooked, wt. . . . .	150
Bread . . . . .	50	Bread . . . . .	50
Oleo . . . . .	25	Oleo . . . . .	25
Cornstarch . . . . .	35	Cornstarch . . . . .	35
Sugar . . . . .	95	Sugar . . . . .	95
Milk . . . . .	240	Milk . . . . .	240

<sup>1</sup> The above diet supplied the following calculated daily average food values: calories 3275; protein 88 gm; fat 139 gm; carbohydrate 417 gm; vitamin A 13,000 I.U.; ascorbic acid 100 mg; thiamine 1.3 mg; riboflavin 2.2 mg and niacin 15.5 mg.

<sup>2</sup> This was generously supplied by Dr. E. J. Czarnetzky of Wilson and Company, Inc., Chicago.

times: 2 oz. each before dinner and supper, 1 oz. at mid-afternoon, and 5 oz. during the evening.

During the experimental period, the carbohydrate diet was consumed for 7 days, the alcohol diet for the next 10 days, and the original carbohydrate diet for the last 6-day period. Beginning with the second day of the diet, 24-hour urines were collected (kept in the refrigerator with 15 ml of glacial acetic acid) and pooled in 48-hour periods for analysis of thiamine, riboflavin, nicotinic acid, N<sup>1</sup>-methylnicotinamide, pantothenic acid, 4-pyridoxic acid, pteroylglutamic acid, tryptophane, creatinine and nitrogen.

Thiamine was measured by a modification of the thiochrome method using a sulfite blank (Mason and Williams, '42). N<sup>1</sup>-methylnicotinamide and 4-pyridoxic acid (as the lactone) were also measured fluorometrically by modifications of the methods of Huff and Perlzweig ('43, '44).

In the microbiological methods used for determination of riboflavin, nicotinic acid, tryptophane, pantothenic acid and pteroylglutamic acid, 40 gm each of glucose and of sodium acetate were used per liter of medium. *Lactobacillus casei* was used (1) for riboflavin assay with this change in the medium of Snell and Strong ('39) and (2) for pteroylglutamic acid assay with a medium similar to that of Mitchell and Snell ('41). The medium of Krehl, Strong and Elvehjem ('43) was employed with *Lactobacillus arabinosus* as the test organism for assay of nicotinic acid and tryptophane. When used for tryptophane analysis, this component of the medium was replaced by 400 µg of nicotinic acid per liter. *Lactobacillus arabinosus* was also used for measurement of pantothenic acid (Skeggs and Wright, '44). All microbiological tests were titrated with 0.1 N NaOH (bromthymol blue as indicator) after incubation at 37°C. for about 65 hours.

Aliquots of the urines were digested with sulfuric acid and 30% hydrogen peroxide for determination of total nitrogen with the use of Nessler's reagent. Creatinine was measured by a simple colorimetric procedure using alkaline picrate re-

agent. A photoelectric colorimeter was used for these readings.

#### RESULTS

The urinary excretion of the substances mentioned above is shown in table 2 for the 2 subjects while on the carbohydrate and alcohol diets. The excretion of creatinine was relatively constant during the experimental period and attests to the completeness of sample collection. Subject S started the experiment at a higher level of nitrogen excretion than did subject B, but later the excretion of nitrogen by the 2 subjects was almost the same. The change from carbohydrate to alcohol diets did not significantly affect the nitrogen excretion of either subject. This is paralleled by the relatively constant tryptophane excretion. Daily weights of the subjects showed less than 1 pound variation from the initial weight during the experiment. No attempt was made to control fluid intakes, and minor variations in urinary volume occurred.

The excretion of thiamine by both subjects increased markedly after changing from the carbohydrate to the alcohol diet, and decreased correspondingly when the carbohydrate diet was resumed. This may be an indication of lower thiamine requirements when carbohydrate is replaced by alcohol in man. Other evidence for a thiamine sparing action of alcohol has been presented for rats (Lowry, '42b) and pigeons (West-erfeld and Doisy, '45). The urinary N<sup>1</sup>-methylnicotinamide was also greatly increased on the alcohol diet in the present experiments, and decreased upon resumption of the carbohydrate diet. Small changes in the nicotinic acid excretion appear to follow the pattern of changes of N<sup>1</sup>-methylnicotinamide excretion on these diets. These results may indicate similar changes in thiamine and nicotinic acid utilization in alcohol metabolism.

The riboflavin excretion of subject S was much higher than that of subject B at the beginning of the experiment. This might be correlated with the higher initial nitrogen excretion of this subject whose diet contained more milk prior to the experiment. Both subjects showed variations in riboflavin

TABLE 2  
Daily urinary excretion of vitamins and other metabolites by 2 subjects on carbohydrate and alcohol diets.

URINARY EXCRETION	SUBJECT	CARBOHYDRATE DIET					ALCOHOL DIET					CARBOHYDRATE DIET				
		Days					Days					Days				
		2-3	4-5	6-7	8-9	10-11	12-13	14-15	16-17	18-19	20-21	22-23				
Thiamine ( $\mu$ g)	B	93	66	66	104	111	111	147	190	98	91	80				
	S	80	70	68	99	111	104	116	118	77	80	60				
N <sup>1</sup> -Me <sup>1</sup> (mg)	B	4.5	3.7	4.5	6.3	5.5	6.4	6.5	6.9	6.0	5.8	4.6				
	S	6.5	5.6	5.0	5.8	7.3	7.5	9.0	10.2	8.5	8.8	4.7				
Nicotinic acid (mg)	B	0.9	0.8	1.1	1.3	1.2	1.4	1.2	1.6	1.1	0.9	0.8				
	S	1.0	0.8	1.0	1.4	1.3	1.2	1.1	1.6	0.9	0.9	0.8				
Riboflavin ( $\mu$ g)	B	340	490	510	750	555	500	540	570	380	480	540				
	S	1180	950	975	1090	700	510	470	550	380	540	600				
4-Pyridoxic acid (mg)	B	1.4	29.6	28.5	18.8	3.5	4.1	4.0	8.0	4.3	35.3	29.3				
	S	4.8	28.0	32.5	20.0	3.6	3.0	2.8	7.3	3.2	34.7	10.0				
PGA <sup>2</sup> ( $\mu$ g)	B	2.7	3.0	2.5	3.3	3.0	2.7	3.2	3.1	2.5	2.5	2.0				
	S	3.4	3.4	3.0	3.5	3.6	3.7	3.3	3.2	3.0	3.0	3.2				
Pantothenic acid (mg)	B	3.1	3.2	2.8	3.4	2.5	2.5	2.6	2.8	2.2	2.5	2.7				
	S	4.8	4.7	4.1	4.8	4.3	3.6	3.9	4.1	3.2	3.6	3.6				
Tryptophane (mg)	B	26	24	23	23	21	24	23	24	22	23	23				
	S	20	20	19	19	24	21	20	21	20	20	20				
Creatinine (gm)	B	2.0	2.0	2.1	2.2	1.9	2.3	2.1	2.1	1.9	2.1	2.1				
	S	2.2	2.2	2.3	2.3	2.5	2.3	2.3	2.3	2.1	2.2	2.2				
Nitrogen (gm)	B	11.2	12.3	12.9	13.3	13.7	14.6	13.2	13.7	11.0	12.8	12.3				
	S	15.9	15.1	14.8	16.3	12.0	14.2	13.6	14.5	13.2	13.4	13.2				
Urine volume (ml)	B	1520	1450	1170	1370	1330	1390	1430	1530	970	1070	960				
	S	1190	1150	1250	1270	1200	1500	1170	1510	1100	1030	1230				

<sup>1</sup> N<sup>1</sup>-methylnicotinamide.<sup>2</sup> Pteroylglutamic acid.

excretion which are not readily interpretable in regard to requirement for alcohol and carbohydrate metabolism. The experimental periods may have been too short to reflect differences, if any, in riboflavin requirements on these diets.

The excretion of 4-pyridoxic acid is an indication of the metabolism of the pyridoxine complex (Huff and Perlzweig, '44; Johnson, Hamilton and Mitchell, '45) but the level of excretion has little meaning in human nutrition at the present time. Johnson et al. ('45) have reported an average daily excretion of 3.5 mg of 4-pyridoxic acid, and in the authors' laboratory a range of 1 to 5 mg per day is usually found for normal subjects. On the initial carbohydrate diet, the excretion of this substance by both subjects started in the normal range and increased to about 30 mg per day. This value decreased on the alcohol diet and approached the normal level. The final carbohydrate diet again resulted in this unexplainable excretion of high levels of the acid. Since these values were obtained by fluorometric analysis, it is possible that some unknown metabolite of the carbohydrate diet was interfering with the analysis. The sugar and cornstarch, which were consumed on the carbohydrate diet, contained insignificant amounts of vitamin B<sub>6</sub>. The cornstarch<sup>3</sup> contained 0.07 µg of vitamin B<sub>6</sub> per gram as measured<sup>4</sup> by the method of Atkin et al. ('43). The 70 gm of cornstarch therefore contributed only about 5 µg of pyridoxine compounds to the diet. Another possible explanation for the high values is that a pyridoxine compound was synthesized in large amounts by intestinal bacteria in the presence of excessive cornstarch and sugar in the diet, but this was not reflected in the excretion of any of the other vitamins.

The excretions of both pteroylglutamic acid and pantothenic acid remained constant during the experimental period and were not affected by changes in the alcohol or carbohydrate contents of the diet.

<sup>3</sup> Powdered Buffalo cornstarch, Corn Products Sales Co., New York.

<sup>4</sup> This analysis was made through the courtesy of Dr. S. H. Rubin of Hoffmann-LaRoche, Inc.



## DISCUSSION

Urinary levels of vitamins and other metabolites are resultants of many processes in the body. Therefore, the increased excretion of thiamine and N<sup>1</sup>-methylnicotinamide which accompanied the isocaloric substitution of alcohol for carbohydrate in the present studies may have been caused by one or more factors. On the basis of our present knowledge of vitamin excretion, these results may be interpreted as suggesting a thiamine and nicotinic acid sparing action of alcohol. The increases in excretion do not appear to be attributable to the breakdown of tissue protein (Sarett, Klein and Perlzweig, '42) since the subjects maintained their weight and showed little change in the excretion of nitrogen and several of the other substances measured. It is possible that liver cell injury (Lowry et al., '42a) may have released enough thiamine and nicotinic acid to account for the extra vitamin excretion, with small nitrogen loss which would not be detectable in the present experiments. However, the excretion of the other vitamins, which should have been released at the same time, did not follow the patterns for thiamine and N<sup>1</sup>-methylnicotinamide.

The thiamine sparing action of alcohol, when this substance is substituted for an equicaloric amount of a thiamine deficient diet for rats, has been shown by Lowry et al. ('42b) by a delayed development of polyneuropathy and death. Similar conclusions were reached by Westerfeld and Doisy ('45) who were able to delay the onset of opisthotonus in pigeons by substitution of alcohol for either fat or carbohydrate in a thiamine deficient ration.

It is difficult to understand how alcohol can exert a thiamine and nicotinic acid sparing action since its metabolism is thought to be linked with that of pyruvic acid, requiring both thiamine and nicotinic acid-containing enzymes (Leloir and Munoz, '38; Westerfeld, Stotz and Berg, '42, '43). It is possible that other pathways of alcohol metabolism may exist. However, even the assumption that alcohol requires no thiamine for its metabolism would not completely explain the following findings. The substitution of alcohol for fat (which

supposedly requires little or no thiamine for its metabolism) has a further thiamine sparing action in pigeons (Westerfeld and Doisy, '45), and the *addition* of alcohol to the thiamine deficient diet of pair-fed rats has a thiamine sparing action (Lowry et al., '42b). Westerfeld and Doisy ('45) suggested that the metabolism of carbohydrate and alcohol together may require less thiamine than does the metabolism of carbohydrate alone. This would be equivalent to a negative thiamine requirement of alcohol.

The indications from the above animal experiments and the present human studies may not be applicable to cases of excess alcohol intake with a low caloric intake of other foods.

#### SUMMARY

The isocaloric replacement of carbohydrate (sugar and cornstarch) by ethyl alcohol in an adequate diet resulted in an increased excretion of thiamine and N<sup>1</sup>-methylnicotinamide in 2 human subjects. No significant changes were found in the excretion of creatinine, nitrogen, tryptophane, pantothenic acid and pteroylglutamic acid.

These data may suggest that the substitution of alcohol for carbohydrate has a thiamine and nicotinic acid sparing action.

The excretion of 4-pyridoxic acid on the carbohydrate diet was increased to unexplainably high levels.

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# GROWTH AND REPRODUCTION OF RATS FED ARMY COMBAT RATIONS<sup>1</sup>

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## INTRODUCTION

Extensive studies have been made of the composition of army combat rations. These analyses for carbohydrate, protein, fat and vitamins indicated that the daily intake was sufficient to supply the daily dietary requirements (QMC studies, '44). Very little work has been done on the feeding of these rations to experimental animals. The requirement for growth of an animal, such as the rat, would be higher for certain essential nutrients than that needed by man for maintenance. Nevertheless, animal feeding experiments might yield information which would be of value in improving the formulation of rations for man.

## EXPERIMENTAL

The combat rations were those used by the U. S. Army and supplied by the Quartermaster Food and Container Institute for the Armed Forces. The C ration had been designed specifically for men in actual combat where no kitchen facilities

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were available. It was packed in 6 small cans. Three cans, 1 for each meal, contained the meat components and offered any one of 10 different varieties, such as chicken and vegetables, ham and lima beans, pork and rice, and meat and spaghetti. The 3 other cans, again 1 for each meal, contained the bread components and included in toto biscuit, beverage powder, sugar, confectionery, pre-mixed cereal and jam.

The 10-in-1 ration was intended to serve troops in all areas where they were in advance of the field kitchen but sufficiently protected to permit small group feeding. It provided food for 10 men for 1 day. Each case of 10-in-1 ration contained 1 of 5 distinct menus, thus permitting each soldier 15 different consecutive meals. The 5 menus contained various components of canned meat items, biscuits, puddings, cereals, jams, vegetables, butter, sugar, milk, beverage powder, and confection (QMC studies).

The K ration was created to provide a ration light in weight, yet containing the essential nutrients. Each meal included approximately 4 ounces of either meat, meat and egg product or cheese spread, together with biscuits, confection, and beverage powder with sugar (QMC studies).

The components of the ration were ground in a meat grinder and thoroughly mixed in a Hobart mixer. Storage of all rations in the refrigerator reduced the possibility of rancidity. When the rations were supplemented with the B-complex, the following levels in milligrams per 100 gm of ration were used: thiamine 0.3, riboflavin 0.3, niacin 2, pyridoxine 0.2, pantothenic acid 2, folic acid 0.025, biotin 0.01, inositol 10, choline 100, and p-aminobenzoic acid 25. In cases where the vitamins were fed by dropper, the amount administered per day was equivalent to that included in 10 gm of ration.

In order to determine the ability of the ration to support reproduction and lactation in rats, the unsupplemented rations were fed to female weanling rats (Sprague-Dawley). Six rats were used in each group, and they received the ration and fresh water *ad libitum*. The animals were kept in separate cages until the tenth week of experiment, then each group

was placed in a large cage. At the end of the thirteenth week, females were mated with males from our stock colony.

The methods used in handling rats on reproduction experiments have been described previously (Sporn et al., '47). The females were remated after a 3-week rest period. The results obtained were presented as the total of both matings (table 1).

TABLE 1

*Growth and reproduction studies on rats fed army rations.*

	RATION			
	K	C	10-in-1	K + $\frac{1}{2}$ gm raw liver/day
Female rats				
% conception	94	100	100	83
% stillborn	20	20	20	17
% survival of young	8	49	29	65
Male rats				
Average weekly wt. gain (gm) at end of:				
Fourth week	18	21	19	..
Seventh week	22	26	22	..

The experiments were repeated with double the number of animals in each group, and approximately the same results were obtained. Since the preliminary results showed K ration to be the poorest in supporting survival of young, raw liver was added to the diet of rats receiving this ration. It had a very pronounced effect in increasing the survival of young born to females fed this diet (table 1).

The inability of rats receiving the combat rations to support the survival of young indicated that the rations might be inadequate in known or unknown nutritional factors. Growth studies were undertaken to examine this possibility, since they constitute a faster method of analysis.

Male weanling rats (Sprague-Dawley) weighing between 35 and 40 gm were used in all growth experiments. They were housed in individual cages and were fed the ration and fresh

water *ad libitum*. Six rats were used in each group. Each series was repeated at least once.

Feeding the K, C, and 10-in-1 rations, respectively, to groups of male rats indicated that normal growth was not obtained (table 1). It is interesting to note that the average gain per week was increased when the rats were kept on experiment for a longer period.

TABLE 2  
*Effect of supplements added to K ration fed to rats.*

GROUP <sup>1</sup>	B VITAMINS	A AND D	CASEIN	SALTS IV	LIVER	AVERAGE GAIN/WEEEK AT END OF 4TH WEEK
						<i>gm/week</i>
1		+				19
2			+			20
3				+		19½
4					½ gm raw liver/day	24
5	Twice weekly by dropper					20
6	In ration	+				23
7	In ration	+			3% W.L.S. <sup>2</sup>	24
8	In ration	+	+		3% W.L.S.	33
9	In ration	+	+	+	3% W.L.S.	36
10	Double level in ration					26
11	Double level in ration			+		25
12	Double level in ration		+			34
13	Double level in ration		+	+		36
14	Double level in ration		+		ethanol extract of liver	37
15	Double level in ration		+	+	ethanol extract of liver	36

<sup>1</sup> Five rats per group.

<sup>2</sup> Whole liver substance.

Since the K ration gave poor growth and was easily prepared for feeding, it was decided to work more intensively with this ration than with the others. Vitamins A and D, as oleum percomorphum diluted 1:4 with corn oil and fed at the rate of 2 drops per week, 5% casein, or 2% salts IV had no effect when added individually to the diet of rats on K ration (table 2, groups 1, 2 and 3). When the B-complex was fed by

dropper, no effect upon growth was noted (group 5). This addition, when mixed in the ration, brought about a growth response equivalent to feeding  $\frac{1}{2}$  gm of raw liver per day (groups 4 and 6). Supplementation with whole liver substance did not produce a response above that observed with the B-complex (group 7).

When K ration was supplemented with the water-soluble vitamins as well as casein,<sup>2</sup> good growth was obtained (group 12). The addition of salts IV produced a small but consistent weight increase when included with these supplements (groups 9 and 13). When an ethanol extract of liver (Jaffé and Elvehjem, '47) was added to this improved diet as a source of unknown factors, no greater gain could be observed (groups 14 and 15).

The marked improvement with casein suggested an amino acid deficiency. With K ration containing a double level of B vitamins as the base of reference, individual amino acids were fed at 0.3% of this diet. The addition of phenylalanine had no effect upon growth; lysine had an inhibitory effect, the rats averaging 5 gm a week less than those on the basal diet; methionine caused an increased growth of 4 gm a week per rat above that for the basal group. When all 3 amino acids were added to the diet, again no effect upon the growth rate was observed. The effects of lysine and methionine seem to counteract each other when fed simultaneously.

On the basis of the information gained from experimentation with the K ration, the other combat rations were supplemented with similar factors. The C ration was not improved with the addition of vitamins A and D (table 3). The B vitamins produced a slight increase in the growth rate, but casein was necessary before fair growth could be obtained with this diet.

In the case of 10-in-1 ration, the water-soluble vitamins produced a small increase in the rate of gain. Even with both vitamins and casein, the growth rate was still suboptimum (table 3).

<sup>2</sup> Smaco brand.



Animals on most of the diets appeared normal at the end of the experimental period. The rats on unsupplemented K and 10-in-1 rations were an exception. Some of them showed a characteristic loss of hair. If they were allowed to continue on the particular diet for longer periods, there was a spontaneous recovery from this condition. Autopsy did not reveal any gross irregularities among any of the animals.

TABLE 3

*Effect of supplementation of army rations upon rat growth.*

RATION <sup>1</sup>	AVERAGE GAIN / WEEK AT END OF 4TH WEEK
	<i>gm/week</i>
C ration alone	21
C ration plus vitamins A and D	21
C ration plus vitamins A and D plus B vitamins	24
C ration plus vitamins A and D plus B vitamins plus 5% casein	33
10-in-1-ration alone	18
10-in-1 ration plus vitamins A and D	18½
10-in-1 ration plus vitamins A and D plus B vitamins	22
10-in 1 ration plus vitamins A and D plus B vitamins plus 5% casein	27

<sup>1</sup> Five rats per group.

The effect of storage upon the nutritional quality of the rations was also investigated. The K, C and 10-in-1 rations which had been returned from overseas were fed to groups of rats. In all cases, results indicated that these rations supported a growth rate comparable to that observed with fresher rations.

#### DISCUSSION

The results obtained from the *ad libitum* feeding of the combat rations to rats indicate that all of the rations were unable to support growth or survival of young in this species. The materials fed represented only a limited sampling since the items included in the rations were changed from time to time. Therefore, samples taken from other manufacturing sources and at different times might give results varying in

some degree from those reported in this instance. Furthermore, the rations were intended primarily for maintenance whereas growth and reproduction were the 2 criteria used in these experiments for evaluating the several rations. The growing animal would have a higher requirement for certain essential nutrients. The experimental data showed that when the animals were fed the rations for some time, a greater gain per week was observed at later periods. This indicates that the rations were better suited to support growth of the older animals, which have a lower requirement for certain nutrients.

Work which appeared recently tends to emphasize the difference in requirements between species. Cox et al. ('47) studied the effect of adding methionine to an enzyme casein hydrolysate fed to rats, dogs and man, and found that the requirement for methionine in man is lower than that for the dog. This was in confirmation of work published by Johnson et al. ('47).

It is seen from the results that none of the combat rations supported good growth or survival of young in the rat. From the studies on growth, it is observed that with the addition of a mixture of the B vitamins and casein, the K ration was improved so that normal growth was obtained.

Similarly, growth of rats upon C ration can be improved by the addition of the B complex and casein. Here again, the effect of casein is most striking. It is possible that the K and C rations are deficient in similar amino acids. Then again, casein may also be supplying unknown factors. Work from this laboratory (unpublished data) indicates that the casein used in this study contains an unidentified factor for the rat.

The 10-in-1 ration differs from the other rations tested. Though a combination of the B complex and casein exerts a beneficial effect, normal growth in the rat was not obtained.

When raw liver was fed as a supplement to the K ration, the results indicated improvement in growth and lactation. This may be attributed to added vitamins and proteins in the liver. It is interesting to note that a rather high level of vitamins in the diet is necessary to produce the maximum

response. It is a fairly well-established fact that best results are obtained when the vitamins are fed in the diet, and not as supplements in concentrate form at certain periods.

Supplementation with vitamins A and D had no effect upon the growth of rats fed any of the combat rations. Salts IV, when fed in addition to the B vitamins and casein, produced a slight stimulation of growth in rats on K ration. This increased growth rate was not large enough to be significant. The liver extract (Jaffé and Elvehjem, '47) was similarly inactive in increasing the rate of growth of the rats.

The phenomenon of loss of hair has been observed by other workers. Spitzer and Phillips ('46) observed a similar loss of hair in rats fed a corn-soybean ration, which was overcome by feeding inositol or biotin. It is believed that this condition is brought about by factors which influence the intestinal flora. The flora is prevented from supplying adequate amounts of some factor, probably biotin. In the case of rats receiving army rations, this condition is overcome without adding the necessary factors to the diet.

Work by Dunn et al. ('47) indicated that the K ration was low in phenylalanine, lysine and methionine, if compared to levels established by Rose as minimum for the rat. When these amino acids were fed as the probable source of activity in casein, only the methionine gave a positive response. Lysine produced an inhibition of growth. They nullified the effect of each other when fed as a mixture. This establishes only methionine as being necessary for increased growth of the rat on K ration. A more intensive study of amino acids as supplements to the K ration is contemplated.

#### SUMMARY

The army combat rations K, C and 10-in-1 were fed *ad libitum* to young growing rats. The materials fed represent only a limited sampling since the items included in the rations have been changed from time to time. Therefore, the samples

taken from other manufacturing sources and at different times might give results varying in some degree from those reported in this instance. Furthermore, growth and reproduction over extended periods of time were the 2 criteria used in these experiments for evaluating the rations. Actually these rations were consumed for the most part by mature men in the field during relatively short intervals of time.

When the K, C and 10-in-1 rations were fed to female rats, it was found that the survival rate of the young born to the females was quite low. The addition of liver to the K ration improved the percentage of survival of young born to females on this diet.

The growth of male rats on these diets was suboptimum. The addition of the B vitamins and casein to the rations substantially improved growth in all cases. Normal growth was obtained when K and C rations were supplemented with casein and vitamins. Results indicate that part of the response to casein with the K ration is due to the methionine content.

#### ACKNOWLEDGMENTS

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# STUDIES WITH MONKEYS FED ARMY COMBAT RATIONS<sup>1</sup>

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TWO FIGURES

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## INTRODUCTION

Extensive studies have been made of the chemical composition of army combat rations, with special emphasis on the vitamin content, as a guide to their formulation and improved methods of storage. Practically no work has been done on the feeding of these rations to experimental animals. In order to guide ration development, it is important to know whether the combination of foodstuffs theoretically capable of providing an adequate diet is actually complete when used for extended periods of time.

Recent work indicates very clearly that the requirements for different vitamins, and probably amino acids, differ considerably depending on the type of ration fed. Thus, the kind and level of carbohydrate and fat may substantially alter the vitamin requirements. Such changes in requirements can only be observed through controlled feeding experiments. This

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The subject matter of this paper has been undertaken in cooperation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The opinions or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the War Department.

paper presents results which have been obtained on feeding army combat rations to rhesus monkeys.

There has been little work to correlate the nutritional requirements of man and monkey. Though the monkey is similar to man anatomically, its nutritional requirements may differ in several respects. Nevertheless, evidence may be obtained which will be of value in planning improvements in the ration.

Studies during the past 6 years have yielded much information regarding the use of monkeys in nutritional experiments. Waisman, Rasmussen, Elvehjem and Clark ('43) described a purified diet which is capable of maintaining monkeys in excellent health for extended periods of time. With the use of this diet, it has been possible to study uncomplicated vitamin deficiencies. Studies on thiamine (Waisman and McCall, '44), riboflavin (Waisman, '44), pyridoxine and pantothenic acid (McCall et al., '46), biotin (Waisman et al., '45), folic acid (Cooperman et al., '46a) and ascorbic acid (Shaw et al., '45) deficiencies have already been reported. Recent work has described an unknown factor which is necessary for continued growth and a normal blood picture in monkeys (Ruegamer et al., '47).

#### EXPERIMENTAL

The housing, care and handling of the monkeys have been described previously (Waisman et al., '43). The army combat rations K, C and 10-in-1 were prepared for feeding at 2-week intervals. The components were ground in a meat grinder, thoroughly mixed in a Hobart mixer and stored in a cold room. The monkeys were fed twice daily, and each feeding consisted of the contents of a full 1-pound butter crock. There was food in the cups at all times.

The army combat rations have been described in a previous publication (Sporn and Elvehjem, '48). The vitamin supplement was fed at the beginning of each day, and the monkeys usually drank this at once. No water was given to them until they had consumed this supplement. When milk was fed, it

was added after the vitamins were finished and before water was given to the animals.

The "mixture of water-soluble vitamins" referred to in the experiments consisted of the following (in milligrams) fed each day: thiamine hydrochloride 1, riboflavin 1, pyridoxine hydrochloride 1, calcium pantothenate 3, nicotinic acid 5, choline chloride 25, biotin 0.02, folic acid 0.1, p-aminobenzoic acid 50, i-inositol 50, and ascorbic acid 25. When individual vitamins were fed, they were administered at the levels noted.

Hemoglobin and differential leucocyte determinations were performed throughout the study. Samples were taken from blood drawn from the marginal vein of the ear. Hemoglobin determinations were made by the method of Evelyn ('36).

## RESULTS

### *C ration*

The C ration supported good growth in monkeys for about a month. At the end of this period, there occurred a decline in weight which lasted about 2 months and became more rapid at the end of the second month. All indications were that the monkeys would die without supplementation. With the addition of a mixture of the water-soluble vitamins, there was an immediate response in weight. When the vitamins were continued as the only addition to the ration, fairly good growth of monkeys was obtained for at least 1 year. The addition of 100 ml of raw milk per day did not improve the rate of growth observed after the supplementation with the vitamins. Typical growth curves of monkeys on C ration are shown in figure 1. Both monkeys appeared normal at the end of the experimental period.

The hemoglobin concentration of the blood was below normal after the monkeys had been on the C ration for 6 weeks. It took 1 to 2 weeks for the hemoglobin concentration to respond to the addition of vitamins to the diet. A maximum hemoglobin concentration was reached, in all cases, within 1 month after supplementation. The hemoglobin remained in



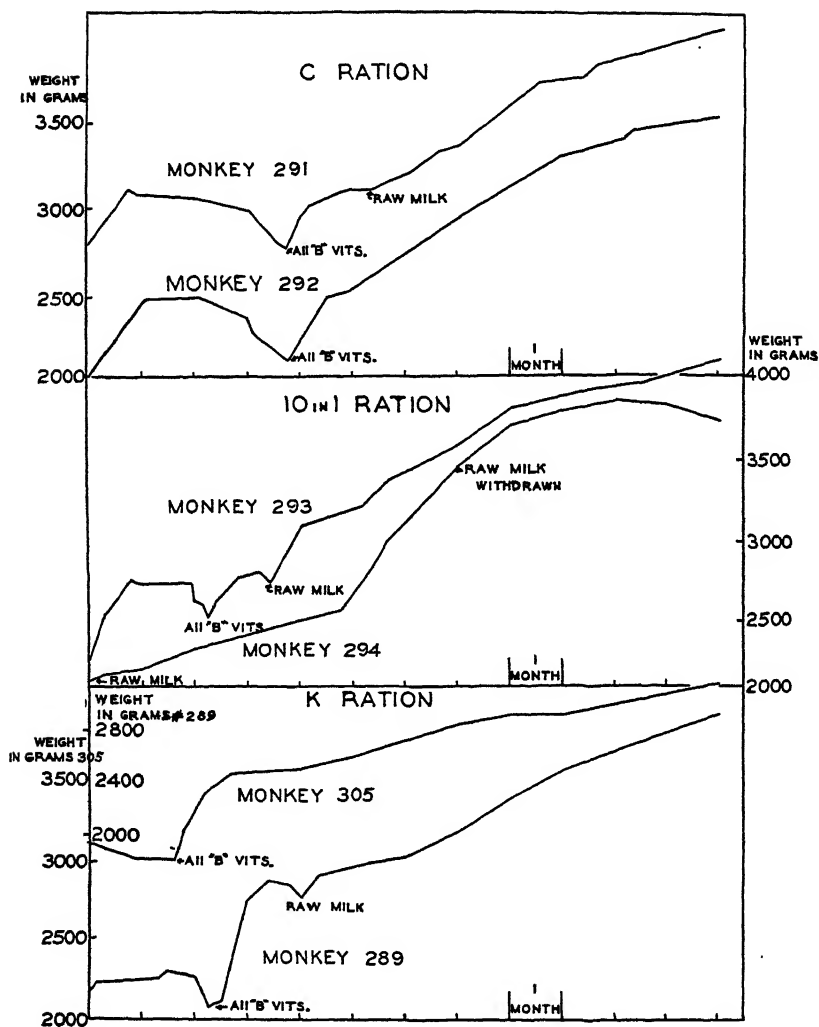


Fig. 1 Typical growth curves for monkeys fed the C, 10-in-1 and K rations. The additions of a mixture of the water-soluble vitamins, as well as 100 ml of raw milk per day, are indicated. Once added to the diet, the supplements were fed daily thereafter.

the normal range for the duration of the experiment, even though it fell slightly about 2 months after supplementation (table 1). The differential leucocyte count was normal at all times.

TABLE 1

*Typical hemoglobin concentrations in monkeys on the C ration.<sup>1</sup>*

	MONKEY NO. 291	MONKEY NO. 292
	gm %	gm %
Start of experiment	14.5	13.5
At the end of 6 weeks	12.5	13.5
Two weeks after vitamin supplementation was started	15.9	16.8
Two months after vitamin supplementation was started	14.2	14.7
After 1 year on experiment	14.0	14.1

<sup>1</sup> Studies in this laboratory on normal growing, young monkeys showed a normal range in hemoglobin concentration of 14–15.5 gm %.

### *Ten-in-one-ration*

The 10-in-1 ration, when fed in the unsupplemented form, produced a slight gain in weight and maintained monkeys for at least 2 months. At the end of this period, there was a sharp decline in weight. The addition of a mixture of the water-soluble vitamins brought about a weight response, but the maximum weight was not greater than the previous maximum attained. The weight did not rise above this point, but was maintained if the vitamin addition to the diet was continued (fig. 1—monkey no. 293).

With the addition of 100 ml of raw milk per day, the weight of the monkeys continued to increase at a normal rate. If this addition was delayed for 1 month, it took some time before this weight response was noted. The 10-in-1 ration plus these additional supplements supported good growth in monkeys for over a year. When the milk was withdrawn, the gain in weight continued for approximately 2 months. At the end of this period, there was a slow but constant decline in weight (fig. 1—monkey no. 294). The appearance of the 5 monkeys on this regimen was quite normal at the end of the experimental period.

The hemoglobin concentration of the blood of monkeys on this diet was always below normal. The addition of raw milk or vitamins did not produce any significant variation in this respect. After 1 year, with both supplements being fed in addition to the 10-in-1 ration, the hemoglobin concentration was approximately 12.0 gm %. The differential leucocyte count was normal throughout the experiment.

### *K ration*

When 9 young rhesus monkeys were fed K ration, there was practically no gain in weight. They were able to maintain themselves on this ration for approximately 2 months. At the end of this period, anorexia developed. Feeding the animals by stomach tube did not alleviate the condition. If supplementation was not started quickly enough, the animals lost weight rapidly and death followed.

When monkeys fed the K ration started to lose weight, a mixture of all the water-soluble vitamins was added as a supplement. The addition produced an immediate weight response. The weight increased at a slow but constant rate for a period of 9 months (fig. 1 — monkey no. 305). When 100 ml of raw milk per day was added to the vitamin supplement, a growth response and a rapid rate of gain were noted. This normal rate of gain lasted over 1 year when both supplements were fed (fig. 1 — monkey no. 289).

The data indicated that of the 3 combat rations tested, K ration was the poorest in supporting growth of monkeys (fig. 2). It was decided to concentrate on this ration to find exactly which factors were producing a response when added to the diet. Monkeys were placed on the unsupplemented ration for about 6 weeks. At that time, they were supplemented with individual vitamins, while the weight and hemoglobin responses were closely followed.

The supplementation procedure consisted of feeding a single vitamin at a time. This was done with animals which had not gained weight for at least a month previously, or were

losing weight rapidly. A response was considered significant if the monkey gained at least 100 gm in 7 to 14 days. Once a vitamin was added to the diet, it was usually fed daily thereafter. Another vitamin was not added to the diet until a definite plateau in the weight curve or a rapid loss of weight was noted.

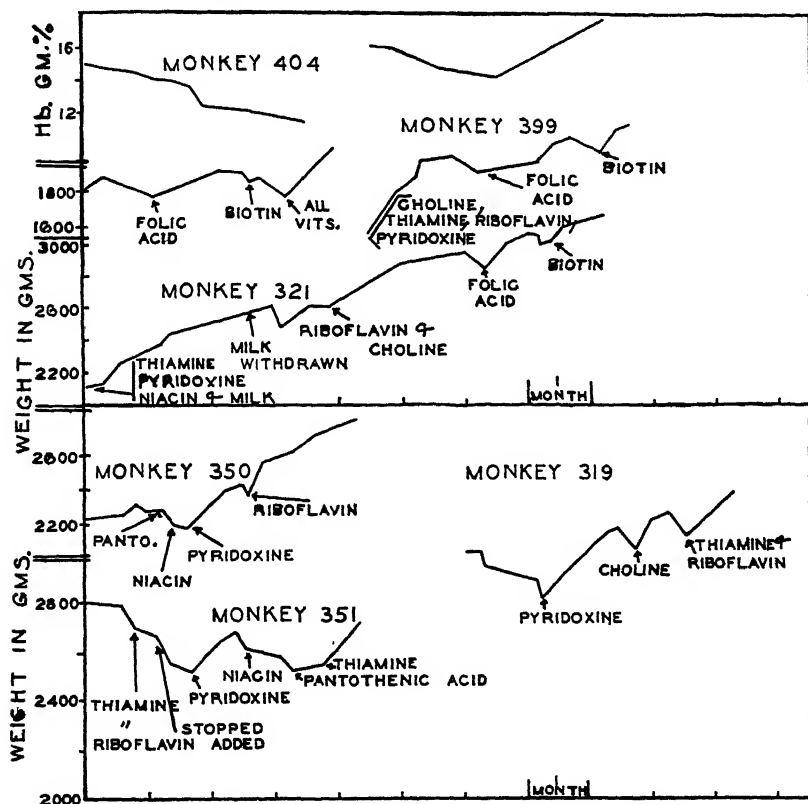


Fig. 2 Typical growth responses of monkeys fed the K ration. Individual vitamin supplementation is shown in various animals.

When pyridoxine was added singly as a supplement to the ration, a sharp weight response was noted. Identical responses were noted in at least 4 different monkeys when pyridoxine was added before or after other vitamins. The weight re-

sponse lasted a short time, and then a loss of weight followed. This type of response occurred with all vitamins active in stimulating growth on K ration.

A mixture of thiamine and riboflavin was added to the diet of monkeys that were losing weight following the original response on B<sub>6</sub>. There was an immediate gain in weight. In other cases, when thiamine or riboflavin was added separately to the vitamin supplement following pyridoxine therapy, there was a similar significant response. When either of these vitamins was added prior to pyridoxine, no increase in weight could be detected following such addition. This indicated that pyridoxine was a limiting factor in these studies.

Choline was added to the vitamin supplement of monkeys following the typical response to pyridoxine. In all cases this addition brought about a definite weight increase. It was tried prior to pyridoxine therapy, and results indicate that the same response could not be observed. It was necessary to have added B<sub>6</sub> present in the diet in order to obtain activity by the addition of choline.

The same results were obtained with biotin. A weight increase was produced by its addition to the diet, but only when supplementary pyridoxine was present. This substantiates the limiting action of pyridoxine.

Niacin and pantothenic acid were added as individual supplements to this ration. They were fed before and after the monkeys received other vitamins. In all cases, little response was detected upon their addition to the diet. Pyridoxine did not alter this condition in contrast to its effect when thiamine and riboflavin were the supplements.

In 1 particular case, a monkey which had received unsupplemented K ration for approximately 2 months was fed milk when anorexia developed. It was necessary to give him a single dose of B<sub>6</sub> (2 mg) in order to prevent further loss of weight and possible death. The milk supplement alone supported a slow rate of gain. The addition of thiamine, which is present in milk in small amounts, was able to produce a weight increase. Additional niacin and vitamin C, both low

in milk, had no effect whatsoever. Pyridoxine, even when fed in addition to milk, brought about a very large and prolonged weight increase.

Thiamine, riboflavin and pyridoxine were fed as supplements to monkeys on K ration from the beginning of an experiment. The diet did not support growth of monkeys, but did extend the maintenance period on the ration. On this regimen, monkeys could survive for at least 3 months. In 1 case, the addition of choline at that time brought about a sharp weight increase.

### *Blood*

The hemoglobin concentration of the blood of monkeys on K ration dropped to approximately 10 gm per 100 ml of blood after the animals had been on the unsupplemented ration for 6 weeks. The addition of a mixture of water-soluble vitamins improved this condition and produced an optimum hemoglobin concentration of 15 gm %. The hemoglobin remained at this optimum level when no further additions were made to the ration, and while the gain of weight was slow.

As stated previously, the addition of milk produced a rapid gain in weight. This caused a drop in the hemoglobin concentration of the blood. Monkeys which had been fed both milk and vitamins as supplements to the ration for a period of 1 year showed a hemoglobin concentration of approximately 12.5 gm %.

Pyridoxine and folic acid were the only vitamins tested which produced a response in hemoglobin. The hemoglobin response was slower than the weight response, but an optimum level was reached in a short time. This same type of response was noted in studies on pyridoxine and folic acid deficiencies on a synthetic ration.

Pyridoxine produced an optimum hemoglobin level which lasted approximately 2 months, after which sub-optimum levels were observed. Folic acid was the only vitamin able to bring the hemoglobin back to the normal concentration.

This effect of folic acid could not be observed unless pyridoxine was present as a supplement to the ration.

The differential leucocyte count was normal throughout the experiments.

### *Autopsy*

One monkey died 1½ months after being placed on K ration. The autopsy revealed extensive hemorrhagic lesions in the colon which extended as far as the appendix. Indications of pneumonia complicated the condition.

Another monkey died in about the same length of time on the K ration. Autopsy disclosed a similar hemorrhagic condition in the colon, as well as a mottled liver containing 60% fat (dry weight). The case was complicated with tuberculosis, lesions being easily detectable in the lungs.

A third animal failed after responding to treatment with individual vitamins; pyridoxine, choline, thiamine and riboflavin had been fed in addition to the K ration. Autopsy revealed extensive intestinal intussusception. This was believed to be the cause of death. Aside from a large, dark spleen and an enlarged liver, all other organs appeared normal.

### DISCUSSION

The results presented in this paper are based on the *ad libitum* feeding of army combat rations to young growing monkeys. It should be emphasized that the materials used represent only a limited sampling since the items included in the rations have been changed from time to time. Therefore, it must be recognized that the samples taken from other manufacturing sources and at different times might give results varying in some degree from those reported in these experiments. One other important consideration is the fact that in these experiments, growth and hematopoiesis covering an extended period of time were the 2 criteria used in evaluating the several rations. Actually, in the field these foods are consumed for the most part by mature men and for relatively short periods of time.

Data obtained from the feeding experiments indicate that none of the combat rations studied could support growth in the monkey for extended periods of time. The C ration produced the best results, since it was able to support a slight gain in weight when fed in the unsupplemented form. It was only necessary to add a mixture of the water-soluble vitamins in order to improve this ration. When this was done, the diet supported fairly good growth of monkeys for over a year.

The 10-in-1 ration needed 2 supplements in order to improve its quality. The addition of a mixture of the water-soluble vitamins produced a weight increase but not as great as in either of the other cases. However, when raw milk plus the vitamins was added to the diet of monkeys on 10-in-1 ration, good growth was obtained over extended periods.

Monkeys fed the K ration died within 2 months. The addition of a mixture of water-soluble vitamins improved the ration to a certain extent, but raw milk was necessary before it was able to support good growth of monkeys.

Eight of the 11 vitamins present in the mixture of these water-soluble factors were tested individually by adding them as supplements to the K ration. It was observed that a significant increase in weight was obtained when either thiamine, riboflavin, pyridoxine, choline, folic acid or biotin was added to the diet. Niacin and pantothenic acid were inactive in this respect.

This particular study is one of the first instances in which monkeys have been fed a ration which seems to be deficient in a number of factors. The type of response observed is similar to that which is obtained with rats on a multiple-deficient diet. A response in weight is noted when a factor is fed in which the diet is deficient, but the weight drops soon thereafter. It is difficult to state whether a response indicates a lack of the factor added, or is an indirect effect of the addition.

The Committee on Food Composition of the Food and Nutrition Board, National Research Council, reported the



analysis of the K ration. Their reported analysis, as well as the recommended allowances are presented:

	N.R.C. ANALYSIS	DAILY ALLOWANCE RECOMMENDED FOR MODERATELY ACTIVE MAN BY N.R.C.
	<i>mg/day unit</i>	<i>mg</i>
Thiamine	1.33	1.5
Riboflavin	2.60	2.0
Niacin	12.7	15.0

From these figures it is seen that the levels of thiamine and riboflavin in the ration should be sufficient to satisfy the requirements of man. However, the results show that these vitamins produced a growth response when added to the ration fed the monkeys.

The National Research Council allowances are usually high enough to be optimum under most conditions. The requirements for thiamine and riboflavin appear to be the same for man and monkey, when figured on the basis of body weight. It must be remembered that the figures in the table represent a daily portion for man, whereas the monkeys were fed *ad libitum*.

It is possible that the levels of vitamins needed on this ration are higher than will suffice ordinarily. It has been shown that the type of carbohydrate, protein or fat may alter the amount of the known vitamins necessary on a particular diet (Luckey et al., '46). Then again, there may be an imbalance of factors in the ration. This has been shown to have an adverse effect in the case of amino acids (Krehl et al., '46).

It is difficult to believe that the growth responses noted on the addition of thiamine or riboflavin are due to the fact that the K ration is deficient in these vitamins. The lack of any specific deficiency signs supports this contention. The freckled dermatitis which is typical of a riboflavin deficiency in the monkey was completely absent. No symptoms of thiamine deficiency as described by Waisman and McCall ('44) could be observed.

The possibility of stimulation of intestinal synthesis is plausible. The addition of these known factors may cause the intestinal flora to produce known or unknown factors in which the ration is low. This would explain the growth response in spite of the fact that the vitamins added were present in the ration in optimum amounts.

The question of poor absorption was eliminated by a study of urinary excretion. The urines of monkeys on K ration receiving added riboflavin and riboflavin plus milk were compared. Greater amounts were secreted in the second case. The fact that an appreciable amount of riboflavin was present in the urine, eliminated poor absorption on this ration as a significant factor.

An interesting observation in these experiments is that pyridoxine is a limiting factor. This vitamin must be present as a supplement to the diet before the effect of 3 of the 4 active vitamins can be noted; it does not fall into the same position as thiamine and riboflavin. Microbiological assays for vitamin B<sub>6</sub> (Rabinowitz and Snell, '47) indicated 0.185 mg per 100 gm of ration. Though the B<sub>6</sub> requirement of the monkey is not definitely known, experience with other animals indicates that this may be a border-line amount.

There seems to be a clear-cut deficiency for folic acid and pyridoxine. The increase in hemoglobin concentration upon the addition of the water-soluble vitamins was due to pyridoxine and folic acid. This was proven with individual vitamin supplementation. They were the only vitamins able to cure deficiency symptoms other than growth. Here again, pyridoxine showed its limiting effect. Folic acid was ineffective in producing a hemoglobin response unless B<sub>6</sub> was present as a supplement to the diet.

Biotin seems to fall into the same category as thiamine and riboflavin. Its beneficial effect is noted only in the presence of supplementary pyridoxine. Though several monkeys developed a loss of hair previous to complete vitamin supplementation, biotin could not be definitely identified as the

curative agent. Its effect may be due to stimulation rather than to an actual deficiency for the vitamin.

In considering choline, it is difficult to determine exactly how this vitamin may be concerned. It was added originally when a fatty liver was noted upon autopsy of 1 monkey which died on the unsupplemented K ration. Choline produced a significant weight response when added to the diet of monkeys. No data are available on the choline content of the ration, and no work has been performed with choline deficiency in monkeys. It may very well be a true vitamin deficiency in the case of this factor.

Intestinal intorsusception has been reported in dogs on a pantothenic acid-deficient diet (McKibbin et al., '40). It was also detected on autopsy of a monkey on K ration, one of the few times this has been observed in the history of the monkey colony at this laboratory. Pantothenic acid was added as a supplement to the ration of other monkeys after this observation was noted, but results indicate that it had no effect.

Niacin proved of little value as a supplement to the K ration. Workers at this laboratory (Cooperman et al., '46b) were unable to produce a true niacin deficiency in monkeys fed a purified ration. The other water-soluble vitamins were not studied extensively enough with the K ration to report on them at this time.

Thiamine, riboflavin and pyridoxine were added as supplements to K ration from the start of an experiment. This extended the maintenance period on this ration for at least another month. It supports the evidence that one of these factors is deficient in the K ration.

The blood picture is an indication of the adequacy of a ration. The hemoglobin concentration of the blood of monkeys on the C ration was within the normal range of 14 to 15.5%, when this ration was supplemented with vitamins. Even with vitamins plus milk, the 10-in-1 ration was not able to support a normal hemoglobin level. There is still a factor lacking in the case of this ration. Evidence indicates that it is not the same factor as reported by Cooperman et al. ('46a), as the

characteristic reversal of the neutrophil-lymphocyte count was not observed.

The K ration, with additional vitamins, supported a normal hemoglobin level when the monkeys grew at a slow rate. When the growth rate was normal, the hemoglobin concentration dropped to a sub-optimal level. Here again, differential leucocyte counts showed that this was not due to a deficiency of the monkey anti-anemia factor.

It must be realized that man and monkey do differ in their nutritional requirements. Clear-cut examples of pyridoxine, pantothenic acid and biotin deficiencies have not been observed in man. These have been produced and studied quite intensively in the monkey. Biotin deficiency, which usually requires the administration of raw egg white or sulfa drug before symptoms are observed, can be produced in monkeys simply by eliminating biotin from the ration. This evidence tends to interfere with correlation between the species.

The monkeys used in these experiments were young animals with little stores of essential nutrients. These fast-growing animals have a higher requirement for such nutrients than would be needed for maintenance. These facts indicate that the rations cannot be rejected on the basis of our experiments. They have been used to maintain healthy men for short periods. Under such conditions, the rations would probably be quite sufficient. If the rations were to be used to support growth or for maintaining depleted individuals, certain improvements would be advisable.

#### SUMMARY

The army combat rations K, C, and 10-in-1 were fed *ad libitum* to young growing monkeys. The materials fed represent only a limited sampling since the items included in the rations have been changed from time to time. Therefore, the samples taken from other manufacturing sources and at different times might give results varying in some degree from those reported in this instance. In these experiments, growth

and hematopoiesis covering an extended period of time were the 2 criteria used in evaluating the several rations.

The results of the experiments carried out under these conditions indicate that none of the combat rations tested could support growth in monkeys. The C ration could be substantially improved by the addition of a mixture of the water-soluble vitamins. The 10-in-1 ration produced normal growth in monkeys when supplemented with the vitamins plus raw whole milk. The K ration required the same supplements in order to support normal growth in monkeys for an extended period of time.

The supplemented C and K rations could support a normal hemoglobin concentration. Normal growing monkeys on the supplemented K and 10-in-1 ration had sub-optimum hemoglobin levels.

Thiamine, riboflavin, pyridoxine, folic acid, biotin and choline were individually active in stimulating growth in monkeys on the K ration. Niacin and pantothenic acid were inactive in this respect. The effect of thiamine, riboflavin and biotin may be indirect, since specific deficiency symptoms are absent. For the growing monkey, K ration seems deficient in pyridoxine and folic acid, and possibly choline as well.

#### ACKNOWLEDGMENT

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# THE SYNTHESIS OF CERTAIN B VITAMINS BY THE RABBIT <sup>1</sup>

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The physiological significance of the B vitamins has been intensively studied in many animal species and it is generally accepted that the mono-gastric animals require a dietary source of most of the B vitamins, while the functional ruminants synthesize them in their digestive tracts. On the other hand, very little work has been done to determine the role of the different B vitamins in the nutrition of the rabbit.

The first indications of a very particular behavior of rabbits in relation to their requirement of a vitamin of the B complex were reported by Passmore ('35). A purified diet supplemented with a yeast preparation was modified to produce a vitamin B<sub>1</sub> deficiency by autoclaving the yeast preparation at 130°C. for 20 minutes at pH 9.5. Three rabbits fed this diet for 40 days gained in weight and it was concluded that vitamin B<sub>1</sub> was synthesized in the alimentary canal. Baglioni ('36) fed 2 rabbits a diet deficient in vitamin B<sub>1</sub>, which consisted of an autoclaved mixture of beans and butter supplemented with calcium lactate, sodium chloride and lemon juice. The animals did not show any symptoms attributable to a vitamin deficiency after ingesting the diet for 65 days, and the urine contained sufficient vitamin B<sub>1</sub> to prevent beriberi in pigeons receiving a polished rice diet. Hogan and

<sup>1</sup> Some of the data were taken from a thesis submitted to the Graduate School by Orlando Olcese in partial fulfillment of the requirements for the degree of Master of Science.



Ritchie ('34) succeeded in raising rabbits when a diet of a semi-purified nature was fed. In a later report (Hogan and Hamilton, '42) it was shown that rabbits grew at the normal rate on simplified rations which contained dried yeast as the source of all water-soluble vitamins.

Wooley and Sebrell ('44) reported that rabbits required niacin for normal growth when fed a purified diet which contained 20% casein. In view of the recent work on the apparent utilization of dietary tryptophane as a source of nicotinic acid, the report of these workers, that an increased growth rate was observed when nicotinic acid was added to a purified diet, is rather surprising. This seems particularly true when little or no growth response was obtained with the rat when nicotinic acid was added to diets also containing 20% casein (Hundley, '47; Schweigert and Pearson, '48).

It was of importance, therefore, to obtain additional data on the requirements of the rabbit for B vitamins. The rate of growth and urinary and fecal excretion of riboflavin and pantothenic acid were determined when diets low in these vitamins were fed. Some studies have also been carried out on the excretion of biotin and pteroylglutamic acid (folic acid) by the rabbit.

#### EXPERIMENTAL

Weanling rabbits of the New Zealand White breed were used for these experiments, and animals of the same sex were equally distributed among the different groups in each experiment. The animals were kept in groups in wire bottom cages and were weighed at weekly intervals.

The composition of the different diets used is shown in table 1. During the first few days on experiment, the simplified diets were mixed with stock ration to facilitate the acceptance of the purified rations. After this period the rabbits accepted the purified diets without any objection. Food and water were provided *ad libitum*.

Metabolism studies were carried out to determine whether or not pantothenic acid and riboflavin were being synthesized by the rabbit. Since the diets did not contain additional biotin

or pteroylglutamic acid, it seemed advisable to determine the balance of these 2 vitamins. Food consumption records were kept and the amounts of pantothenic acid, riboflavin, biotin and folic acid in the diets determined.

TABLE 1  
*Composition of the diets.*

DIET NO.	1	1a	2	2a	3	4
	<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>
Dextrin	37.5		37.5		37.5	
Cerelose <sup>1</sup>		37.5		37.5		37.5
Beet pulp	30	30	30	30	30	30
Cellulose	3	3	3	3	3	3
Peanut oil	8		8		8	
Corn oil <sup>2</sup>		8		8		8
Commercial casein	18		18		18	
Purified casein <sup>3</sup>		18		18		18
A and D oil	0.5	0.5	0.5	0.5	0.5	0.5
Salts IV <sup>4</sup>	3	3	3	3	3	3
Supplements per 100 gm of diet						
	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>
Mixed tocopherols	50	50	50	50	50	50
Choline chloride	200	200	200	200	200	200
Niacin	20	20	20	20	20	20
Inositol	10	10	10	10	10	10
2-methyl 1,4-naphthoquinone	0.075	0.075	0.075	0.075	0.075	0.075
Pyridoxine	0.7	0.7	0.7	0.7	0.7	0.7
Thiamine	0.7	0.7	0.7	0.7	0.7	0.7
Riboflavin	0.7	0.7	0.7	0.7	0.7	
Calcium pantothenate			1.5	1.5	0.5	1.5

<sup>1</sup> Glucose monohydrate.

<sup>2</sup> Mazola.

<sup>3</sup> Labco.

<sup>4</sup> Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E.B., J. Biol. Chem., 138: 459, 1941.

For the metabolism studies, the rabbits were placed in individual wire bottom metabolism cages for a period of 72 hours; the feces were retained by a screen, while the urine passed through and was collected in dark bottles containing acetic

acid. These bottles were changed every day and kept in a refrigerator, and at the end of the third day the contents of the 3 bottles from each rabbit were mixed together. The volume of urine collected during the 3-day periods ranged from 150 to 350 ml; however, water was sometimes spilled from the drinking containers and the volume collected was therefore increased. The urine collected from each rabbit in each period was made up to a volume of 1000 ml with water to give a more uniform concentration of the vitamins. The urine samples were neutralized and appropriate aliquots were taken for assay.

The feces, after being collected, were dried for 24 hours at 60°C., weighed, and finely ground. Samples were then taken for determinations of pantothenic acid, riboflavin and biotin.

Pantothenic acid was determined by the method of Neal and Strong ('43) after liberation of the vitamin by autoclaving and enzymatic digestion followed by filtration at pH 4.6. A slight modification was made in the medium by replacing the Vitab supplement with synthetic vitamins. Riboflavin was determined by the method of Snell and Strong ('39). The vitamin was liberated by autoclaving the samples with 0.1 N HCl, and after cooling, the samples were filtered at pH 4.6. Biotin was determined with *Lactobacillus arabinosus* as the test organism by the method described by Wright and Skeggs ('44). The fecal samples were autoclaved with 6 N H<sub>2</sub>SO<sub>4</sub> for 2 hours to liberate the vitamin. Folic acid was determined by the method of Teply and Elvehjem ('45) with *S. faecalis R* as the test organism.

#### RESULTS AND DISCUSSION

In order to determine the performance of rabbits fed different levels of pantothenic acid, the first experiment was carried out with 3 groups of 6 rabbits each receiving diets which contained additions of 0, 0.5, and 1.5 mg of Ca pantothenate per 100 gm of feed (diets 1, 3 and 2, respectively, table 2). During the 50 days on experiment the animals grew normally and no symptoms of a vitamin deficiency were ob-

served in the rabbits fed the pantothenic acid-low diet. The average gains for the 50-day period were similar, 1292, 1227 and 1266 gm, respectively, for the 3 groups. These results indicated that dietary pantothenic acid is not required by the rabbit and that the small differences in the growth observed among the 3 groups were not due to dietary treatment, but to variance within the same population.

Since evidence of a vitamin deficiency was not produced in the first experiment, another series of experiments was conducted to obtain further information. Two groups of 5 rabbits each were used. One group was fed a pantothenic acid

TABLE 2

*Effect of dietary treatment on the growth of rabbits.*

GROUP	NUMBER OF RABBITS	DIET	DAYS ON EXPERIMENT	BODY WEIGHT GAIN
				gm
1	6	1	49	1292
2	6	2	49	1266
3	6	3	49	1227
4	5	1a	112	2117
5	5	4	112	2164
6	5	1a	105	2235
7	5	2	105	1850
8	5	4	105	1847

deficient diet (no. 1a), and the other, a riboflavin deficient diet (no. 4). More highly purified ingredients were used in order to reduce the amount of vitamins contributed by the diets fed. The pantothenic acid content of the pantothenic acid deficient diet was found to be 0.31  $\mu$ g per gram. The riboflavin content of the riboflavin deficient diet was 0.33  $\mu$ g per gram. Diets this low in pantothenic acid and riboflavin would not support normal growth in rats. Unna ('40) and Henderson et al. ('42) have shown that 80  $\mu$ g of pantothenic acid is the minimum daily requirement of the young rat. Assuming a daily consumption of 20 gm of feed by the rat, diet 1a would provide only 6.2  $\mu$ g of pantothenic acid. Wagner

et al. ('40) and Mantering et al. ('41) reported that the minimum daily requirement of riboflavin for the rat is between 18 and 30  $\mu$ g. With 20 gm of daily food consumption, diet 4 would provide only 6.6  $\mu$ g of riboflavin per day.

This experiment was carried out during the summer time and the hot weather was not favorable for optimum performances. Consequently, the animals had a low food intake; however, the ingestion of the diets low in pantothenic acid and riboflavin failed to produce any vitamin deficiency symptoms. After 55 days, one of the rabbits on the pantothenic acid deficient diet developed diarrhea and died. This animal had been gaining weight normally and its death is not attributed to a pantothenic acid deficiency. The growth of the other animals in both groups was satisfactory. The rabbits were fed these diets for 16 weeks. At this time the average weight of the rabbits on the pantothenic acid deficient diet was 3196 gm and the average weight of the rabbits on the riboflavin deficient diet was 3130 gm.

Starting with the sixth week, some of the rabbits tended to eat the hair of the other animals. Areas devoid of hair appeared on some of them, apparently not attributable to spontaneous alopecia. This was evinced by the growth of new hair when the rabbits were placed in individual cages.

At the end of the 16-week period the animals were changed to rations containing 1% sulfasuxidine. The sulfasuxidine was added at the expense of the cerelose. While on this dietary regimen, 2 of the rabbits in each group developed diarrhea and died. None of the animals showed any symptoms that could be definitely attributed to a pantothenic acid or riboflavin deficiency. Low gains were obtained after the addition of sulfasuxidine to the diets, due in part to the fact that the rabbits were reaching maturity. However, 2 rabbits on the low pantothenic acid diet and 1 on the low riboflavin diet showed a loss of weight.

Urine and fecal collections were made for 3 different periods for each rabbit before and after the feeding of sulfasuxidine. The results obtained for the metabolism

studies are summarized in table 3. Each figure for the pantothenic acid deficient rabbits is the average obtained for 3 collections from each of 4 rabbits; for the riboflavin deficient animals each figure is the average for 3 collections from each of 5 rabbits. The data obtained on the amount of riboflavin excreted demonstrate clearly that this vitamin is being synthesized by the rabbit. The animals fed the riboflavin deficient diet ingested an average of 11.1  $\mu\text{g}$  of riboflavin daily and excreted 127.1  $\mu\text{g}$  before the feeding of sulfasuxidine. This

TABLE 3

*Ingestion and excretion of riboflavin, pantothenic acid and biotin by rabbits when fed low-pantothenic acid and low-riboflavin diets before and after the addition of sulfasuxidine (values expressed in  $\mu\text{g}$  per 24 hours).*

DIET NUMBER	DESCRIPTION	BEFORE FEEDING SULFASUXIDINE				AFTER FEEDING SULFASUXIDINE			
		Intake	Excretion			Intake	Excretion		
			Urine	Feces	Total		Urine	Feces	Total
Riboflavin balance									
4	low-riboflavin	11.1	112.9	14.2	127.1	11.0	109.6	31.0	140.6
Pantothenic acid balance									
1a	low-panto- thenic acid	8.5	34.7	15.2	49.9	8.8	28.0	27.6	55.6
Biotin balance									
4	low-riboflavin	0.27	1.64	..	..	0.33	1.08	..	..
1a	low-panto- thenic acid	0.28	0.99	..	..	0.29	0.75	..	..

represents an 11-fold increase in the amount excreted as compared to the amount ingested. Similar results were obtained for the pantothenic acid studies. For the group receiving the low pantothenic acid diet the excretion of this vitamin was 49.9  $\mu\text{g}$  per day as compared with an ingestion of 8.5  $\mu\text{g}$ . The amount excreted was, therefore, 6 times higher than the amount ingested. Since biotin assays were not made of all the fecal samples, the data obtained for only the urine are presented in the table. However, the urine values alone indi-

cate that biotin was synthesized. The daily ingestion of biotin by the rabbits fed diets 1a and 4 averaged 0.28 and 0.27  $\mu\text{g}$ , with corresponding excretions in the urine of 0.99 and 1.64  $\mu\text{g}$ , respectively. The daily ingestion of folic acid by the rabbits fed diets 1a and 4 averaged 1.1  $\mu\text{g}$ . The average daily excretion of this vitamin in the urine by the 5 rabbits receiving diet 1a was 30.6  $\mu\text{g}$ , and by the 5 receiving diet 4, 34.1  $\mu\text{g}$ , which greatly exceeded the intake.

After the feeding of sulfasuxidine, there was a slight increase in the amount of riboflavin excreted by the rabbits fed the low riboflavin diet, as compared with the excretion before the feeding of the sulfa drug. The amount of pantothenic acid excreted by the rabbits fed the low pantothenic acid diet after the feeding of sulfasuxidine also increased slightly as compared to the amount excreted before the feeding of the drug. Similarly the excretion of biotin was not reduced when sulfasuxidine was fed (table 3). The ingestion of this level of sulfasuxidine did not, therefore, appear to have affected the amounts of these vitamins synthesized by the rabbit.

The excretion of folic acid, however, showed a marked decrease when sulfasuxidine was fed. After the feeding of the sulfa drug, the daily urinary excretion of folic acid was reduced from an average of 30.6 to 1.30  $\mu\text{g}$  for the animals fed diet 1a, and from 34.1 to 0.83  $\mu\text{g}$  for the ones fed diet 4. Several of the rabbits died after developing a severe diarrhea when fed sulfasuxidine. All these facts suggest that if a deficiency of a vitamin was responsible for the deaths, it must have been a deficiency of folic acid or/and other factors(s).

A third series of experiments was carried out in order to compare the growth of 5 rabbits fed a diet low in pantothenic acid and 5 fed a diet low in riboflavin, with the growth of a control group receiving both of the vitamins. They received diets 1a, 4 and 2a, respectively. The animals were kept on these diets for 15 weeks, and none of them showed any symptom either of a vitamin deficiency or diarrhea. The gains were good with all 3 diets and the group fed the low-pantothenic acid diet made somewhat better gains than the other two. It

is of interest to point out that after termination of these experiments, 2 of the does fed the riboflavin-low diet produced young, thereby indicating that not only will these diets support growth but reproduction as well.

Metabolism studies were carried out also with these rabbits and the excretion of pantothenic acid, riboflavin and biotin determined. The purpose was to verify the results of the first studies and at the same time to compare the ingestions and

TABLE 4

*Ingestion and excretion of riboflavin, pantothenic acid and biotin by rabbits receiving low-pantothenic acid, control and low-riboflavin diets (values expressed in  $\mu\text{g}$  per 24 hours).*

GROUP	DIETARY TREATMENT	INTAKE	EXCRETION		
			Urine	Feces	Total
Riboflavin balance					
6	low-pantothenic acid	407.3	419.5	36.0	455.5
7	control	416.3	365.7	33.2	398.9
8	low-riboflavin	17.0	239.3	25.8	265.1
Pantothenic acid balance					
6	low-pantothenic acid	17.2	99.8	45.1	144.9
7	control	869.4	425.2	37.6	462.8
8	low-riboflavin	790.5	449.4	36.2	485.6
Biotin balance					
6	low-pantothenic acid	0.56	2.14	1.35	3.49
7	control	0.57	2.71	1.55	4.26
8	low-riboflavin	0.52	1.96	1.23	3.19

excretions of the rabbits fed the low-riboflavin and low-pantothenic acid diets with the ingestions and excretions of the control group. The collections were made during the fall season of the year and, as might be expected, the ingestion and excretion were much higher than in the second series conducted during the summer season. Collections were made beginning with the fifth week through the eighth week of the experimental feeding periods.

The results of this study are summarized in table 4, each figure representing an average of 15 collections (3 collections for



each of 5 rabbits). The amounts of the vitamins excreted did not vary appreciably for each of the 3 collections made; consequently, only the average for all collections is presented. The average daily excretions of riboflavin were: for the animals fed the low-pantothenic acid diet, 455.5  $\mu\text{g}$ ; for those fed the control diet, 398.9  $\mu\text{g}$ ; and for the ones fed the low-riboflavin diet, 265.1  $\mu\text{g}$ , with corresponding riboflavin intakes of 407.3, 416.3 and 17.0  $\mu\text{g}$  per day, respectively. These data show, therefore, that a high amount of synthesis occurred when the low-riboflavin diet was ingested. For the other 2 groups fed additional riboflavin, the excretion approximated the intake.

The average daily excretions of pantothenic acid were: for the rabbits receiving the low-pantothenic acid diet, 144.9  $\mu\text{g}$ ; for the rabbits receiving the control diet, 462.8  $\mu\text{g}$ ; and for the ones receiving the low-riboflavin diet, 485.6  $\mu\text{g}$ , with corresponding intakes of 17.2, 869.4 and 790.5  $\mu\text{g}$ , respectively. There was a significant synthesis of pantothenic acid by the rabbits fed the low-pantothenic acid diet. The previous findings, therefore, were corroborated with these studies. Proportionately more riboflavin was synthesized than pantothenic acid, as in the previous study.

The total biotin excreted per day in the case of the animals fed the low-pantothenic acid diet was 3.49  $\mu\text{g}$  as compared to an intake of 0.56. For the control groups, the total biotin excreted amounted to 4.26  $\mu\text{g}$  per day with an intake of 0.57, and for the group fed the low-riboflavin diet the total excretion was 3.19  $\mu\text{g}$ , with an intake of 0.52.

The fact that a high degree of riboflavin and pantothenic acid synthesis was observed when the diets low in these respective vitamins were fed, while the excretion approximated the intake when additional vitamin supplements were included in the diet, suggests either that the best conditions for the synthesis of the vitamins are met when the ration is low in those particular vitamins or that they are destroyed when fed in high amounts. This fact is not surprising since

destruction of riboflavin occurred when it was fed in large amounts to ruminants (Pearson and Schweigert, '47).

The data presented in this work indicate that growing rabbits either have a very low requirement or do not require a dietary source of either pantothenic acid or riboflavin. As for the mechanism by which the absorption of the synthesized vitamin takes place, there are 2 possibilities. The first is that the vitamins synthesized by the flora of the intestinal tract are absorbed directly from the cecum and colon. The second possibility is that, due to the normal night coprophagy shown by the rabbit, the vitamins synthesized in the cecum are absorbed in the duodenum after the ingestion of the feces. It has been demonstrated that rabbits reconsume the feces as voided from the body (Morot, 1882; Madsen, '39; Taylor, '39, '40; and Eden, '40). Taylor ('39) has shown that by the normal function of night coprophagy in the rabbit, very large amounts of feces are reingested. He found that between one-third and one-half of the stomach contents is normally of fecal origin. The suggestion that coprophagy serves as a means of facilitating absorption of the vitamins synthesized in the intestinal tract is supported by the observations of several investigators (Steenbock et al., '23; Dutcher and Francis, '24; Smith et al., '25; Cowgill et al., '25; Salmon, '25; and Kennedy and Palmer, '28) that rats on B vitamin deficient diets showed improved growth when allowed access to their own feces. Although the rabbits used in the present study were kept in cages fitted with wire screened bottoms, no special techniques were employed to prevent coprophagy.

Although dextrin was used in the first experiments and cerelese in subsequent ones, no direct evidence for the comparative effect of these carbohydrates on the amount of synthesis of B vitamins was obtained since no balance studies were carried out when the dextrin diets were used. Dextrin apparently is more favorable than are the simpler carbohydrates for the synthesis of B vitamins by the rat (Guerrant and Dutcher, '34; Mannering et al., '44; Schweigert et al., '45); however, a deficiency of neither pantothenic

acid nor riboflavin was produced in the present study when cerelese was used as the main carbohydrate source. The inclusion of beet pulp may have favored conditions for vitamin synthesis. Nielsen and Elvehjem ('42) have shown that the ingestion of low-riboflavin and low-pantothenic acid diets is not favorable for the synthesis of biotin. It is apparent, therefore, from the present work that the rabbit is capable of synthesizing large amounts of at least certain B vitamins.

#### SUMMARY

1. Pantothenic acid and riboflavin are not essential dietary constituents for the growing rabbit. Diets containing as low as 0.31  $\mu\text{g}$  of pantothenic acid and 0.33  $\mu\text{g}$  of riboflavin per gram induced normal growth in rabbits. No symptoms of a vitamin deficiency were shown by the animals after ingestion of these diets for 112 days.

2. When balance studies were carried out with 2 groups of animals fed the low-riboflavin diet with a daily intake of 11.1 and 17.0  $\mu\text{g}$ , the corresponding total excretions were 127.1 and 265.1  $\mu\text{g}$  of riboflavin per day. Two groups of rabbits fed a pantothenic acid deficient diet ingested daily 8.5 and 17.2  $\mu\text{g}$  of pantothenic acid, and excreted 49.9 and 145.0  $\mu\text{g}$ , respectively.

3. Data obtained on the ingestion and excretion of biotin and folic acid indicate that these vitamins are also synthesized in this species.

4. When 1% of sulfasuxidine was added to the diet, no symptoms specific for a pantothenic acid or riboflavin deficiency appeared, but 5 out of 10 rabbits developed diarrhea and died.

5. Balance studies showed that with the addition of the sulfasuxidine to the diet, only the excretion of folic acid was decreased to a significant extent.

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# MINERAL METABOLISM STUDIES IN DAIRY CATTLE

## III. MANGANESE METABOLISM IN THE LACTATING BOVINE<sup>1</sup>

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ONE FIGURE

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Studies dealing with the nutritional significance and the metabolism of manganese in the bovine have been impeded by the difficulty encountered in preparing basal diets sufficiently low in this element.

Although the liver and kidneys appear to be the main sites of manganese storage (von Oettingen, '35), and ingested manganese effects a temporary rise in the blood level which rapidly returns to normal (Reiman and Minot, '20), most of the manganese ingested by animals appears to be excreted. Kobert (1883), Cahn (1884), Harnack and Schrieber ('01), Barger ('06), Reiman and Minot ('20), Greenberg et al. ('43), and Skinner and Peterson ('30) have demonstrated that ingested manganese is almost entirely excreted in the feces. Several reports indicate that small quantities of the ingested element are absorbed into the blood, but are rapidly lost via

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the liver and bile (Bargero, '06; Reiman and Minot, '20; Richards, '30; Greenberg et al., '43). Only extremely minute quantities of manganese have been detected in the urine (Kobert, 1883; Reiman and Minot, '20; Greenberg et al., '43).

Very low levels of manganese were found in cows' milk: 0.000004% (Richards, '30); 0.000003% (McHargue, '24); and 0.000002% (Archibald, '41; Archibald and Lindquist, '43); however, Archibald and Lindquist ('43) were able to increase the amount 2.5-fold by feeding  $\text{MnSO}_4$  and their results indicated that the cow may store a reserve of this element during the pasture season. Kemmerer and Todd ('31), using similar treatment, were unable to affect the manganese content of cow and goat milk. The poor-quality semen and lowered fertility of bulls receiving a low manganese ration were improved by supplementing the diet with this element (Lardy et al., '42).

#### EXPERIMENTAL

The data on manganese metabolism reported here were derived from the balance trials reported previously (Reid et al., '47). Three weekly balance trials were conducted during the first 5 months of lactation at regular intervals with each of 8 Holstein and 4 Guernsey cows, comprising 4 feed groups.

The general feeding plan provided 1 lb. of timothy-clover hay and 3.5 lb. of corn silage per 100 lb. body weight daily and concentrates in sufficient quantity to provide 110% of the Morrison feeding standards ('39). The 4 groups received the following: group I, the basal concentrate mixture (consisting of ground corn, 30 parts; rolled oats, 30 parts; linseed meal, 28 parts; wheat bran, 11 parts and  $\text{NaCl}$ , 1 part); group II, basal concentrate mixture plus 3% of  $\text{CaCO}_3$  (c.p.); group III, basal concentrate mixture plus 3% of a mixture of  $\text{CaCO}_3$  (c.p.) and  $\text{MnSO}_4$  (c.p.) (proportions of Ca and Mn same as in Mico<sup>4</sup>); group IV, basal concentrate mixture plus 3% of Mico.<sup>4</sup>

<sup>4</sup>Mico has the following percentage composition with respect to the elements listed: calcium 33.0, magnesium 2.0, manganese 0.2, iodine 0.045, copper 0.025, zinc 0.01 and cobalt 0.002.

The method used to determine manganese in all materials was essentially that outlined by Willard and Greathouse ('17) with modifications allowing measurement with an Evelyn photoelectric colorimeter. Table 1 shows the mean manganese content of the feeds ingested and the feces excreted by the 4 groups of cows during 9 trials each.

TABLE 1

*Mean manganese content of concentrate mixtures, hay, silage and feces during 9 trials (per cent).*

	CONCENTRATE FEEDS				MIXED HAY	CORN SILAGE	FECES			
	I	II	III	IV			I	II	III	IV
Mean	.0063	.0058	.0111	.0121	.0066	.0036	.0142	.0136	.0186	.0201
Standard Error	.0000	.0000	.0000	.0001	.0004	.0001	.0002	.0004	.0010	.0008

## RESULTS AND DISCUSSION

Figure 1, showing the range of daily quantities of manganese ingested and excreted in the feces, points out the dependence of the amount of manganese excreted in the feces upon the quantity ingested. These data would indicate that lactating cows retain about the same quantity of the ingested manganese, regardless of the amount ingested, over the range 622.4 to 1325.6 mg daily. A correlation coefficient of  $0.958 \pm 0.014$  existed between the intake and fecal excretion of manganese. On the basis of these data, the expected quantity of manganese retained by lactating cows receiving diets similar to those used in this study may be calculated from a known intake by the following equation:

$$Y = 0.92452X - 85.5 \pm 57.4 \text{ mg}$$

where, Y = mg Mn excreted in feces daily

X = mg Mn ingested daily

then, X - Y = mg Mn retained daily

It is not known whether the same relationship holds or whether the same equation may be applied under other experimental conditions. Factors affecting the retention of man-



ganese by cows have not been studied to any great extent. A similar straight line relationship was observed between the intake and fecal excretion of manganese in our calculations of the data presented by Chornock et al. ('42) for rats on high manganese diets.

Since no manganese was found in reasonable quantities of urine and since only traces were detected in the milk of animals receiving supplemental manganese (as previously reported by Archibald, '41, and Archibald and Lindquist, '43),

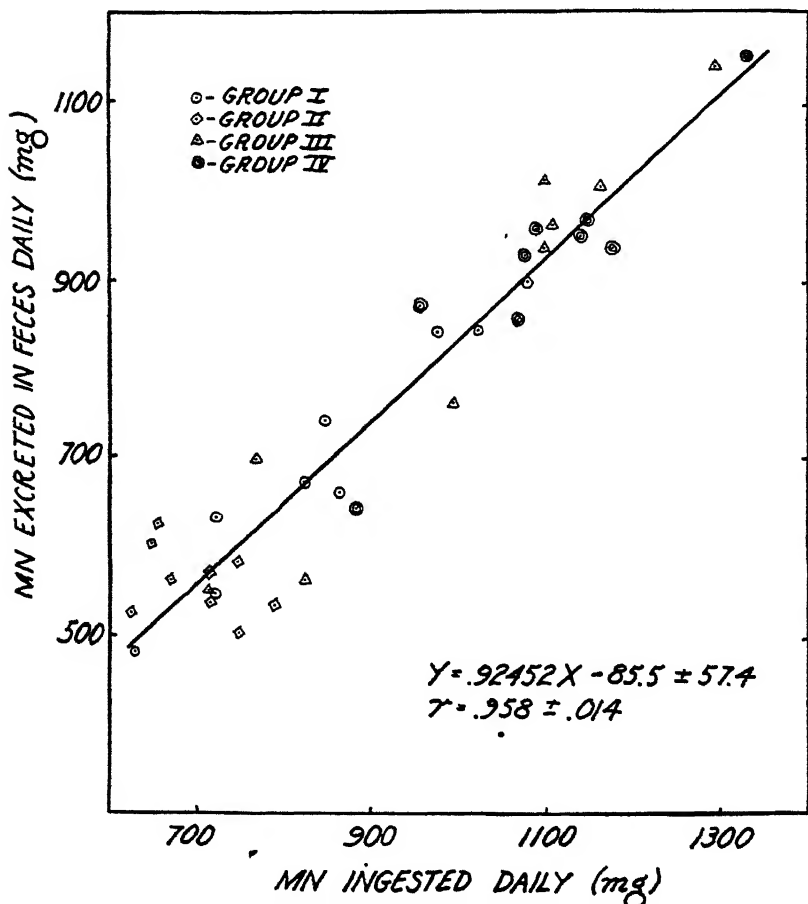


Fig. 1 The relationship of the quantity of manganese ingested to that excreted in the feces.

the quantity of manganese retained daily is practically equivalent to the daily manganese balances for these animals. On the basis of these figures, the occurrence of a negative manganese balance in lactating cows appears remote since it would be extremely difficult to formulate an otherwise normal diet low in manganese for cows. The basal ration employed in this study supplied ample quantities of the element as judged from the great proportion of the intake which was excreted. These data suggested that a lactating cow retains approximately  $154.4 \pm 9.8$  mg of manganese daily.

Manganese added to the rations as  $\text{MnSO}_4$  appeared to be utilized equally as well as manganese derived from feed stuffs.

The addition of supplemental calcium to the rations of the animals of groups II, III and IV, and of supplemental iodine, magnesium, copper, cobalt, zinc and iron to the ration of group IV cows did not significantly influence the quantity of manganese retained. The means and their standard errors of the amount of manganese used by the groups were: group I,  $148.4 \pm 12.5$ ; group II,  $139.2 \pm 26.9$ ; group III,  $155.0 \pm 20.5$  and group IV,  $175.0 \pm 17.1$  mg daily.

#### SUMMARY

A study was made of the metabolism of manganese in 12 cows at 3 intervals each during the first 5 months of lactation.

Lactating cows retained  $154.4 \pm 9.8$  mg manganese daily; about the same quantity of manganese was used regardless of the quantity of the element ingested. The amount of manganese excreted in the feces was found to be directly proportional to the amount of manganese ingested in the range of 622.4 to 1325.6 mg daily; the correlation coefficient for this relationship was  $0.958 \pm 0.014$ . An equation is presented which allows the quantity of manganese excreted daily (and, conversely, the amount retained) to be calculated when the daily intake is known for cows receiving diets similar to those used in this investigation.

The supplementation of the ration with calcium and various trace elements did not appear to influence significantly the amount of the ingested manganese retained.

Manganese added to the ration as  $\text{MnSO}_4$  was utilized equally as well as that provided by the feed materials.

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# INFLUENCE OF NUTRITIONAL FACTORS ON SKELETAL ATROPHY FROM DISUSE AND ON NORMAL BONES OF MATURE RATS<sup>1</sup>

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A previous paper (Armstrong, Knowlton and Gouze, '45) reviewed the problem of skeletal atrophy from disuse and described the effects produced by the administration of an androgenic and of an estrogenic hormone on the composition of the bones of normal and paralyzed limbs of rats. Injections of estradiol dipropionate, at 1 dosage level, were found to result in a reduction of the bony atrophy from disuse and to increase the ash content and amount of bone tissue in the humeri of the normal limbs. Similar studies and results have been presented by Gardner and Halvorsen ('46) who worked with mice. Choline was found by Riley, McCleary and Johnson ('45) not to affect the atrophy of paralyzed limbs of rats, and Landoff ('42) has investigated the atrophy of bones of rabbits caused by immobilization of a limb and by experimentally induced inflammations of the knee joint.

The present study was undertaken in order to obtain data as to the effects of certain nutritional factors on the degree of atrophy occurring in paralyzed limbs. At the same time, an opportunity was also afforded to collect data relative to the influence of the nutritional variations studied on the bones of

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normal limbs of mature animals. There is a wealth of knowledge pertaining to the effects of dietary deficiencies of minerals on the skeleton of young animals, and Sherman and coworkers (Sherman and Booher, '31; Sherman and Sherman, '38; Van Duyne et al., '41; Sherman et al., '41) have investigated extensively the beneficial effects on growth and calcium storage of the feeding, beginning at an early age, of diets more than adequately rich in calcium. There is little published information, however, on the effects produced on the skeleton by nutritional variations imposed on mature subjects aside from the indirect evidence obtained from balance studies. Bauer, Aub and Albright ('29) found that a low intake of calcium resulted in a resorption of the bony trabeculae of the humeri in 4 cats. Incidental to the main purpose of the present study are presented additional data on the effects of estradiol dipropionate on the bones of normal and paralyzed limbs and evidence that sesame oil is not inert in its effects on the skeleton.

#### METHODS

The Wesson ('32) salt mixture and modifications of this salt mixture as shown in table 1 were used to prepare diets of different calcium, phosphorus and protein contents according to the recipes given in the same table. The ingredients of the salt mixtures for diets 2 and 3 were adjusted so that their content of all minerals, except for a deficiency of calcium or phosphorus, was as close as possible to that of the complete mixture. The Cellu-Flour was added to the deficient salt mixtures in order to equalize on a weight basis the mineral contents common to the 3 salt mixtures.

The dry ingredients of the diets were mixed and the melted fat, into which the liver concentrate and choline hydrochloride were dispersed, was then added and the whole thoroughly stirred in a mechanical dough mixing machine. The diets were prepared in 4 kg batches and stored in the frozen state until used. Table 2 shows the calcium, phosphorus and nitrogen contents of representative batches of diets. The calcium determinations were made by the method of Clark and Collip

TABLE 1  
*Composition of salt mixtures and diets.*

Ingredient	SALT MIXTURES			DIETS		
	Complete (Wesson's) (Diets 1, 4, 5, 6)	Calcium deficient (Diet 2)	Phosphorus deficient (Diet 3)	Diets 1-5	Diet 6	
	gm	gm	gm	gm	gm	gm
NaCl	52.5	77.7	52.5	Sucrose 400	540.5	
KCl	60.0	27.8	100.0	Sucrose-vitamin mixture <sup>1</sup> 200	200	
KH <sub>2</sub> PO <sub>4</sub>	155.0	213.8		Lactalbumin (Borden's no. 15-42) 180		
Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub>	74.5			Fat (Spry) 169	208.5	
CaCO <sub>3</sub>	105.0		177.1	Salt mixture <sup>2</sup> 40	40	
Other ingredients of Wesson salt mixture	102.7	102.7	102.7	Liver concentrate (Wilson's fraction D) 10	10	
Cellu-Flour		127.7	117.4	Choline-HCl 1	1	
Total	549.7	549.7	549.7	Total 1000 <sup>3</sup>	1000 <sup>4</sup>	

<sup>1</sup> Sucrose 1000 gm, thiamine-HCl 10 mg, pyridoxine-HCl 10 mg, riboflavin 15 mg, calcium pantothenate 20 mg.

<sup>2</sup> Complete salt mixture used in diet 1 (control), diet 4 (calcium enriched), diet 5 (phosphorus enriched), and diet 6 (protein deficient); calcium deficient salt mixture used in diet 2, and phosphorus deficient salt mixture used in diet 3.

<sup>3</sup> Plus 6.87 gm CaCO<sub>3</sub> in diet 4 (calcium enriched); plus 8.67 gm KH<sub>2</sub>PO<sub>4</sub> in diet 5 (phosphorus enriched).

<sup>4</sup> Plus 1.82 gm KH<sub>2</sub>PO<sub>4</sub> to substitute for phosphorus present in lactalbumin of diets 1-5.

('25) and the phosphorus analyses by the method of Fiske and Subbarow ('25) applied to solutions of the ash of the diets. Calcium lactate was added as a fixative in the ashing of the calcium deficient diets for phosphorus analysis. Nitrogen was determined by a modification of the method of Ma and Zuazaga ('42).

Male rats of the Sprague-Dawley strain, with weights of 240-250 gm when delivered, were fed commercial dog biscuit

TABLE 2  
*Analyses of diets.<sup>1</sup>*

TYPE OF DIET AND NUMBER	NO. BATCHES ANALYZED	CALCIUM	PHOSPHORUS	NITROGEN
		%	%	%
Adequate: 1	13	0.548 ± 0.0050	0.420 ± 0.0027	2.29 ± 0.016
Calcium de- ficient: 2	5	0.015 ± 0.0017	0.409 ± 0.0041	2.27 ± 0.011
Phosphorus de- ficient: 3	4	0.511 ± 0.0123	0.054 ± 0.0026	2.28 ± 0.014
Calcium en- riched: 4	2	0.796 and 0.801	0.416 and 0.450	2.29 and 2.33
Phosphorus enriched: 5	2	0.512 and 0.515	0.524 and 0.511	2.33 and 2.31
Protein de- ficient: 6	4	0.540 ± 0.0028	0.408 ± 0.0192	0.085 ± 0.0038

<sup>1</sup> Data are shown as means ± the standard error of the means except in cases in which all results are shown.

until they attained the weights shown in table 3 when their age was not less than 115 days. In order to permit the development of disuse atrophy of 1 humerus, certain nerves from the cords of the brachial plexus were severed on the right side. Using aseptic technique, the median, radial, ulnar, musculocutaneous, subscapular, axillary, thoracodorsal and supra-scapular nerves were exposed by splitting the pectoralis major muscle with a hemostat, and these nerves were severed as they passed through the axilla. The animals of the several

TABLE 3

*Treatment and postoperative observations.<sup>1</sup>*

GROUP AND NO. OF ANIMALS	TYPE OF DIET AND TREATMENT	WEIGHT AT OPERATION	POSTOPERATIVE GAIN IN WT.	DAILY FOOD CONSUMPTION
		<i>gm</i>	<i>gm</i>	<i>gm</i>
1 (39)	Operated control diet 1	264 $\pm 1.8$	41 $\pm 3.4$	11.0 $\pm 0.21$
2 (32)	Calcium deficient diet 2	272 $\pm 1.8$	28 $\pm 3.6$	10.8 $\pm 0.15$
3 (26)	Phosphorus deficient diet 3	270 $\pm 2.0$	11 $\pm 2.5$	10.2 $\pm 0.31$
4 (20)	Calcium enriched diet 4	264 $\pm 0.92$	32 $\pm 2.8$	11.2 $\pm 0.30$
5 (20)	Phosphorus enriched diet 5	266 $\pm 1.1$	31 $\pm 3.0$	10.2 $\pm 0.14$
6 (21)	Protein deficient diet 6	268 $\pm 1.2$	-66 $\pm 1.5$	5.9 $\pm 0.15$
7 (23)	Food restricted <sup>2</sup> diet 1	264 $\pm 1.4$	-37 $\pm 1.6$	6.0
8 (20)	Unoperated control diet 1	272 $\pm 1.2$	82 $\pm 3.4$	14.8 $\pm 0.32$
9 (23)	Food restricted sesame oil alternate days <sup>3</sup> diet 1	271 $\pm 1.4$	-7 $\pm 1.5$	8.9 $\pm 0.03$
10 (23)	Estradiol D. P. alternate days diet 1	267 $\pm 1.2$	-13 $\pm 4.6$	8.9 $\pm 0.31$
11 (23)	Sesame oil alternate days diet 1	273 $\pm 1.8$	22 $\pm 3.5$	10.1 $\pm 0.24$

<sup>1</sup> Data are shown as means  $\pm$  the standard error of the means.<sup>2</sup> Daily food intake restricted to 6.0 gm, the mean daily food consumption of group 6.<sup>3</sup> Daily food intake restricted to 9.0 gm, the mean daily food consumption of group 10.



groups were fed the diets for 21 days under the conditions and with the treatments indicated in table 3. Each animal was housed in an individual cage and daily determinations of food intake were made. The animals in groups 7 and 9 which were, respectively, the controls for groups 6 and 10, were restricted in food intake as noted in the footnotes to table 3. The animals in groups 9, 10 and 11 received subcutaneous injections of 0.1 ml sesame oil or 2.5  $\mu$ g estradiol dipropionate in 0.1 ml sesame oil as indicated in table 3. All animals were given 3-4 drops of an oil concentrate of vitamin A and calciferol by dropper twice weekly to supply vitamins A and D. In order to prevent the animals chewing the paralyzed extremity, it was necessary to cut short the incisor teeth twice weekly. The animals were sacrificed and the humeri, after prolonged boiling of the fore-limbs in water, were dissected free of soft tissue. The bones were dried and rendered fat-free by continuous extraction in a Soxhlet apparatus for 48 hours with an equal part mixture of alcohol and ether. After the bones were dried and weighed, they were ashed to constant weight in platinum dishes in a muffle furnace at 700°C.

#### RESULTS AND DISCUSSION

The dietary regimens and other experimental conditions employed, the mean initial body weights of the animals, their mean post-operative gain in weight and their mean daily food consumption are shown in table 3. Table 4 is a presentation of the mean results obtained with the humeri. The percentage ash contents of the bones are expressed in relation to the dry, fat-free weights. The results pertaining to the total dry, fat-free and total ash weights of the humeri are expressed per 100 gm of initial body weight of the individual animals. These results are reported in this manner in order to permit a comparison, between the groups, of the mass of bone and of bone mineral in the humeri. This device is employed on the valid assumption that the initial dry, fat-free weights and ash weights of the bones were directly related to the initial body weights. The degrees of atrophy shown in the last column of

TABLE 4

*Effects of dietary variations and of estradiol dipropionate on humeri of normal and paralyzed limbs of rats.<sup>1</sup>*

GROUP	HUMERI OF NORMAL LIMBS			HUMERI OF PARALYZED LIMBS			
	Ash	Dry, fat-free wt. per 100 gm body weight	Ash wt. per 100 gm body weight	Ash	Dry, fat-free wt. per 100 gm body weight	Ash wt. per 100 gm body weight	Degree of atrophy
	%	mg	mg	%	mg	mg	%
1	67.47 ± 0.107	83.88 ± 0.763	56.71 ± 0.490	66.03 ± 0.142	72.60 ± 0.864	47.93 ± 0.605	13.7
2	65.97 ± 0.0833	76.08 ± 0.568	50.12 ± 0.374	62.49 ± 0.181	56.11 ± 0.474	35.08 ± 0.320	36.8
3	65.83 ± 0.123	78.31 ± 0.608	51.57 ± 0.401	63.42 ± 0.208	60.80 ± 0.835	38.20 ± 0.467	31.2
4	68.06 ± 0.134	82.68 ± 0.757	56.25 ± 0.522	67.26 ± 0.112	72.53 ± 0.828	48.78 ± 0.528	12.1
5	67.40 ± 0.154	82.10 ± 0.752	55.33 ± 0.418	66.35 ± 0.131	71.60 ± 0.699	47.51 ± 0.702	14.4
6	68.36 ± 0.132	68.96 ± 0.562	47.08 ± 0.359	67.03 ± 0.190	58.31 ± 0.721	39.09 ± 0.511	29.6
7	67.43 ± 0.0836	75.20 ± 0.668	50.71 ± 0.470	66.42 ± 0.122	65.66 ± 0.629	43.61 ± 0.480	21.4
8	68.14 ± 0.095	81.58 ± 0.911	55.58 ± 0.805	68.16 ± 0.140	81.46 ± 0.964	55.51 ± 0.581	...
9	68.08 ± 0.100	73.65 ± 0.720	50.14 ± 0.496	66.63 ± 0.126	62.47 ± 0.729	41.62 ± 0.504	25.0
10	68.79 ± 0.135	81.83 ± 0.718	56.07 ± 0.639	67.57 ± 0.189	73.55 ± 0.824	49.70 ± 0.622	10.4
11	67.91 ± 0.0872	80.00 ± 0.731	54.31 ± 0.466	66.90 ± 0.109	69.18 ± 0.728	46.28 ± 0.479	16.6

<sup>1</sup> The data are shown as means ± the standard error of the means.

table 4 were calculated from the differences between the mean total ash weight of the humeri of the paralyzed limbs and the average of the mean ash weights of the humeri of group 8. Table 5 is a compilation of significance ratios<sup>2</sup> derived from the mean results in table 4 and is presented to aid in the interpretation of differences between mean results in table 4.

<sup>2</sup> A difference of means is usually considered statistically significant if the significance ratio is 2.0 or greater than 2.0.

There is an indication of an increased rate of new bone formation (hypertrophy) in the humeri of the normal limbs of animals with unilateral brachial paralysis, as is shown by a comparison of the results obtained with the normal humeri of group 1 with the results obtained with the corresponding

TABLE 5  
*Significance ratios.*<sup>1</sup>

	% ASH		DRY, FAT-FREE WT. PER 100 GM BODY WEIGHT		ASH WEIGHT PER 100 GM BODY WEIGHT	
	Normal	Paralyzed	Normal	Paralyzed	Normal	Paralyzed
Group 1 compared with						
Group 2	11.1	15.4	8.2	16.7	10.6	18.8
Group 3	10.1	10.4	5.7	9.8	8.1	12.7
Group 4	3.4	6.8	1.1	0.06	0.6	1.0
Group 5	0.4	1.6	1.7	0.9	2.1	0.4
Group 6	5.2	4.2	15.7	12.7	15.8	11.2
Group 7	0.3	2.1	8.5	6.5	8.8	5.6
Group 8	4.7	.	1.9	.	1.2	..
Group 9	4.2	3.2	9.8	9.0	9.4	8.0
Group 10	7.7	6.5	2.0	0.8	0.8	2.0
Group 11	3.2	4.9	3.7	3.0	3.5	2.1
Group 6 compared with						
Group 7	6.0	2.7	7.1	7.7	6.1	6.4
Group 9 compared with						
Group 7	5.0	1.2	1.6	3.3	0.8	2.8
Group 10	4.2	4.1	8.0	10.1	7.3	10.1
Group 10 compared with						
Group 11	5.5	3.1	1.8	4.0	2.2	4.4

<sup>1</sup> Difference of mean results divided by the square root of the sum of the squares of the standard errors of the means.

humeri of group 8. If this effect is real, the indication is that bone of a lower-than-normal degree of calcification was deposited in the normal humeri of group 1. This last suggestion is derived from the fact that although the dry, fat-free and total ash weights of the normal humeri of group 1 exceeded those of group 8 the percentage of ash in the normal humeri

of group 1 was significantly lower than that of the humeri of group 8. This evidence for hypertrophy of the humeri of the normal limbs is not in agreement with that of Riley, McCleary and Johnson ('45) who, however, used younger rats than those employed in this study.

The feeding of diets which were reduced to the lowest practicable content of calcium or phosphorus but which were otherwise adequate, obviously resulted in a reduction of the percentage of ash, the dry, fat-free and the total ash weights of the humeri of the normal limbs (group 1 compared with groups 2 and 3). It is also obvious that the atrophic process which occurred in the humeri of the paralyzed limbs was accentuated by diets greatly deficient in calcium or in phosphorus. The effects produced on the humeri of both the normal and paralyzed limbs by calcium deprivation appear to be more severe than those caused by a lack of phosphorus.

The effect of a calcium-enriched diet, containing *circa* 0.8% calcium (group 4) instead of *circa* 0.55% calcium present in the diet fed to the animals of group 1, was to increase the percentage of ash of the humeri of both limbs. However, the dry, fat-free and the ash weights of neither humeri were significantly affected by the diet of higher calcium content. These results indicate a limited ability of mature rats, in contrast to younger animals, to store extra calcium when an adequate diet is enriched with this element. There was no certain effect on either of the humeri resulting from a phosphorus-enriched diet (group 5) containing 0.51% phosphorus, instead of the 0.42% present in the diet fed to the animals of group 1.

The effect of a diet severely deficient in protein (nitrogen content 0.08% instead of 2.2%) was to increase slightly, but to a statistically significant degree, the percentage of ash in the humeri of the normal and paralyzed limbs (group 6). The total quantity of bone in the humeri of both limbs was, however, markedly reduced by protein starvation as is seen by comparing the results for dry, fat-free and ash weights with those obtained with group 1.

Group 7 was employed because of the fact that the animals of group 6 spontaneously limited their daily food intake to an average of 5.9 gm per day. On this account, part of the effects noted in the humeri of group 6 could have been due to a reduced intake of minerals and of calories by the animals. The diet fed to the animals of group 7 was the same as that fed to the animals of group 1 but was restricted to 6.0 gm per day per animal.

The results obtained from group 7 when compared with those of group 1 show that the percentage of ash of the humeri of the normal limbs was not affected, and that the effect on the percentage of ash of the humeri of the paralyzed limbs was at the borderline of statistical significance. However, the dry, fat-free and the ash weights of both humeri were reduced by partial starvation. This result could have been effected by an increased rate of bone resorption, thus producing osteoporotic bones. The results obtained with group 6 compared with those of group 7 show that the effects of protein deficiency on the skeleton are more severe than those which are produced by partial starvation equivalent to that experienced by the animals fed the protein deficient diet.

The animals of group 9 whose food intakes were less than their requirements but not as severely restricted as those of the members of group 7, nevertheless suffered more severely deleterious effects on their humeri than those experienced by the animals in the latter group. While no explanation can be offered for the mechanism of the anomalous results, they appear to be related to those for the sesame oil injections received by the members of group 9. Similar deleterious effects on the bones of normal and paralyzed limbs were found in group 11 which also received sesame oil injections but whose food intake was not restricted. These effects of sesame oil on the skeleton are additional evidence that this substance is not metabolically inert. Bruce and Tobin ('40) showed that daily intraperitoneal injections of 0.25 to 1.0 ml sesame oil interfered with weight gain of male rats whose initial body weights were about one-half those of the animals used in this study.

Evidence was also presented to show that injections of the alcohol-insoluble fraction of sesame oil reduced the rate of growth of the tibial diaphysis of suckling rats, and that the treatment prolonged the life of adrenalectomized immature rats and that of adrenalectomized lactating animals. Tobin ('41) found that sesame oil injections prolonged the life of adrenalectomized pregnant rats.

Group 9 is the proper control group for the animals receiving estradiol dipropionate (group 10). A comparison of the results obtained with these 2 groups shows that the effect of the administration of estradiol dipropionate was to reduce greatly the bony atrophy occurring in both humeri of the animals in group 9. This effect of estradiol dipropionate on bone metabolism is also seen when the results obtained with group 10 are compared with those of group 11; this effect is especially marked in the case of the humeri of the paralyzed limbs. It is probable that more markedly beneficial effects on skeletal disuse atrophy would have been obtained from the administration of estradiol dipropionate had the substance not been dissolved in sesame oil and had it not interfered with food consumption. These effects of estradiol dipropionate on bone furnish a direct verification of the effect of estrogenic hormones on the skeleton as deduced by Reifenstein and Albright ('47) from metabolic studies with cases of osteoporosis in the human.

#### SUMMARY

The effects of certain dietary alterations and of injections of sesame oil and estradiol dipropionate on the composition of the humeri of normal and denervated limbs of mature rats have been determined.

Severe calcium or phosphorus deficiencies caused a reduction in percentage of ash and in amount of bone in the humeri of the normal limbs. Both types of mineral deficiencies accentuated the atrophic process occurring in the humeri of the paralyzed limbs. These deleterious effects on bone were more marked in the case of calcium deprivation than in lack of phosphorus.

An increase of the calcium content of an adequate diet by 43% resulted in an increase of the ash content of the humeri of both limbs but produced no other significant effects on the bones. An increase of the phosphorus content of an adequate diet by 23% had no demonstrable effects on the bones of either limb.

Severe restriction of the daily intake of an adequate diet resulted in an atrophy of the humeri of the normal limbs and in an increase of the disuse atrophy of the paralyzed limbs. These deleterious effects were increased in animals receiving the same daily intake of minerals in a diet with a very low protein content.

Evidence was obtained indicating that the injections of sesame oil had a deleterious effect on the humeri of normal limbs and accentuated the atrophy of the humeri of the paralyzed limbs.

The administration of estradiol dipropionate in a dosage of 2.5  $\mu$ g on alternate days was found to decrease the atrophy of the humeri of the normal limbs caused by the combination of sesame oil injections and food restriction, and to decrease the atrophy of the humeri of the paralyzed limbs.

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# EFFECT OF PROTEIN INTAKE ON THE BONES OF MATURE RATS<sup>1</sup>

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The finding (Armstrong, '48) that severe restriction of the protein content of the diet of mature rats produced marked effects on the humeri suggested the present study of the influence of different levels of protein intake on the bones of such animals. While there is considerable information (Conner and Sherman, '36; Osborne and Mendel, '26; Quinn et al., '29) dealing with several phases of the influence of protein intake on skeletal growth of young rats, there appears to be little published knowledge with regard to the effects produced by protein deficient diets on the bones of mature animals.

## METHODS

Diets containing respectively 4.5, 13.5, 9.0 and 18.0% lactalbumin as the source of protein<sup>2</sup> with the composition given in table 1 were prepared according to methods previously described (Armstrong, '48). Additional sucrose was employed, on an equal weight basis, as a replacement for protein in the 3 diets of lower protein content. The difference in the calcu-

<sup>1</sup>Supported by a grant from the Graduate School of the University of Minnesota. This paper was constructed from a thesis submitted by Miss Estremerera to the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the Master of Science degree.

<sup>2</sup>In addition to the small amount of protein contributed by the liver extract.

lated caloric contents<sup>3</sup> of diets 1 and 4, the 2 diets with the largest discrepancy in energy content, is 0.5%.

Male rats of the Sprague-Dawley strain, with weights of 240–260 gm when received, were fed commercial dog biscuit until their weights were within the range 260–265 gm when they were started on the dietary regimens. The 4 diets were fed to correspondingly numbered groups of animals, each consisting of 23 rats, for a period of 30 days. All animals were

TABLE 1  
*Composition of diets per kilogram.*

DIET	1	2	3	4
	<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>
Lactalbumin — Borden's 15-42	180	135	90	45
Sucrose	400	445	490	535
Sucrose + vitamins <sup>1</sup>	200	200	200	200
Fat — (Spry)	169	169	169	169
Wesson's salt mixture	40	40	40	40
Liver extract				
(Wilson's fraction D)	10	10	10	10
Choline hydrochloride	1	1	1	1
Calories (calculated)	4,674	4,666	4,659	4,652

<sup>1</sup> Each 1000 gm of diet contained 2 mg thiamine hydrochloride, 2 mg pyridoxine hydrochloride, 3 mg riboflavin, and 4 mg calcium pantothenate.

either 99 or 113 days of age when the feeding of the synthetic diets was begun and individuals of the 2 age groups were distributed equally among the 4 groups. The principle of pair-feeding was employed in quadruplicate with sub-groups of 4 rats, each animal receiving a diet of different protein content, in order that the mean daily food consumption by the members of the 4 main groups should be equal. The "pace-maker" was almost always the animal receiving the diet of

<sup>3</sup> Calculated from the following caloric equivalent per gram: sucrose 3.94 Cal., fat 9.3 Cal., and lactalbumin 4.1 Cal. It seems possible from information relative to the composition of the lactalbumin used that the assumption that the caloric content of this material is 4.1 Cal. per gram errs on the high side. If this is a fact, it would have the effect of making more nearly equal the caloric contents of the diets.

lowest protein content. After the animals were sacrificed the dry, fat-free and ash weights of the humeri were determined by methods previously described (Armstrong, '48). The ash of the humeri of 4 animals each from groups 1 and 4 was dissolved in hydrochloric acid and analyzed for calcium (Clark and Collip, '25), and for phosphorus (Fiske and Subbarow, '25). The livers were weighed and sections of the organs from representative animals prepared for histologic examination. It was later realized that the animals, when started on the experimental regimens, were nearly, but not quite, at the end of the age period of measurable growth in length of humeri (Armstrong, '46). It thus became of interest to determine whether there were differences, among the groups, in length of these bones. Fortunately, the ashed bones, most of which had been retained, were fused and could be handled. Their length was determined to the nearest 0.1 mm with a vernier micrometer.

TABLE 2

*Observations pertaining to the animals and results with regard to the humeri.<sup>1</sup>*

	PROTEIN CONTENT OF DIET			
	18.0% (Group 1)	13.5% (Group 2)	9.0% (Group 3)	4.5% (Group 4)
Initial body wt. (gm)	262 ± 0.85	262 ± 0.78	262 ± 0.83	262 ± 0.47
Final body wt. (gm)	316 ± 3.4	302 ± 3.4	286 ± 4.4	248 ± 2.8
Daily food intake (gm)	12.1 ± 0.18	12.0 ± 0.17	12.0 ± 0.19	11.9 ± 0.23
Dry, fat-free wt. of humeri (gm)	0.2274 ± 0.00273	0.2251 ± 0.00260	0.2187 ± 0.00232	0.2093 ± 0.00264
Ash wt. of humeri (gm)	0.1537 ± 0.00148	0.1523 ± 0.00165	0.1483 ± 0.00134	0.1405 ± 0.00165
Calculated ash content (%)	67.65 ± 0.307	67.66 ± 0.26	67.82 ± 0.34	67.21 ± 0.36
Length of humeri (mm)	26.27 ± 0.0784	26.33 ± 0.15	26.00 ± 0.106	25.65 ± 0.160

<sup>1</sup> The data are shown as means ± the standard error of the means.

## RESULTS AND DISCUSSION

Table 2 shows the results. All data referring to the humeri are the means of the average results obtained with the 2 bones from individual animals in each group.<sup>4</sup> The data pertaining to the humeri are not recorded in relation to the initial body weight, as in a previous report (Armstrong, '48), because the mean initial body weights of the animals in the 4 groups were identical with small variations from the means.

TABLE 3

*Difference between mean results of experimental groups and those of control group and tests for probable significance of difference of the means.*

GROUP	DRY, FAT-FREE WT. OF HUMERI		ASH WT. OF HUMERI		LENGTH OF HUMERI	
	Diff. <sup>1</sup>	S. R. <sup>2</sup>	Diff. <sup>1</sup>	S. R. <sup>2</sup>	Diff. <sup>1</sup>	S. R. <sup>2,3</sup>
	%		%		%	
2	—1.01	0.61	—0.92	0.63	+0.23	0.35
3	—3.83	2.4	—3.51	2.7	—1.03	2.0
4	—7.96	4.77	—8.59	5.96	—2.36	3.48

<sup>1</sup> Difference of mean results expressed as a percentage of the mean result obtained with the control group (group 1).

<sup>2</sup> S. R. = Significance ratio.

<sup>3</sup> Substitution of S. R. with Fisher's "T" does not affect the interpretation.

Table 3 is a presentation of the differences between the results obtained with group 1 (control) and with each of the 3 experimental groups expressed as percentages. This table also includes the values of the significance ratios <sup>5</sup> to aid in the interpretation of significance of differences between the mean results of the control and experimental groups.

It will be noted that the weight gain of the animals varied in the same order as the protein content of the food, even though all animals received essentially equal caloric intakes during the experimental period. An interesting result which

<sup>4</sup> Group 1, length of humeri—18 animals; group 4, length of humeri—17 animals. In all other cases, 23 animals are represented in the means.

<sup>5</sup> Difference of means divided by the square root of the sum of the squares of the standard errors. A difference of means is usually considered statistically significant if the significance ratio is 2 or greater than 2.

emphasizes this point is the fact that the animals of group 1 gained 54 gm, on an average, while those receiving a diet containing 4.5% protein lost, on an average, 14 gm. The difference in energy intake over the 30-day period between groups 1 and 4, taking into consideration their food consumption and the calculated caloric equivalents of the 2 diets, is 36 Calories, which could account for only 4-5 gm of weight difference (as fatty tissue) between the animals of these 2 groups. Forbes and coworkers ('35, '40) have also shown, with growing and with mature rats, that the weight gain and energy disposition of animals receiving equal total caloric intakes are dependent on the protein content of the diet. These workers, however, did not employ diets containing less than 10% protein. They observed a diminished loss of energy in the feces by the animals receiving the higher amounts of protein, but the metabolized energy decreased in the reverse order. A diminished total heat production by the animals receiving the higher amounts of protein was observed which, by elimination, was attributed to a lowered spontaneous activity since neither the basal heat production of the animals of the several groups nor the estimates of the specific dynamic action of the various diets were different (Forbes et al., '35). More recently Forbes and Swift ('44) have presented evidence that the specific dynamic action (S.D.A.) of foodstuffs fed as a mixture is not the sum of the specific effects of the components. While it is not possible to state whether the S.D.A. of the food fed group 1 of this study was significantly less than that fed the other groups, this possibility is suggested by the work of Forbes and Swift ('44), and this point would bear further investigation. It would also be of interest to determine whether the animals receiving the diets of lower protein content actually expended more energy in activity than the animals receiving the higher amounts of protein. The observations of Slonaker ('31) in regard to the effect of protein intake on spontaneous activity are not entirely pertinent to the present study since he employed diets of 10, 14, 18, 22 and 26% protein content fed *ad libitum* to rats. He did not observe any regular order of ac-

tivity of his animals, as determined in revolving cages, to be correlated with the protein content of the diet.

The results pertaining to the humeri show that the bones of the animals consuming the diet containing 13.5% protein (group 2) had only slight and statistically insignificant lower dry, fat-free and total ash weights than those of the control group. The humeri of the animals of groups 3 and 4 clearly suffered a significant reduction in total dry, fat-free and ash weights. It appears, from the results obtained with group 3, that 9.0% protein in the diet is near the level of dietary protein required to maintain the skeleton of rats, such as those used in this study. There appears (table 3) to have been, in group 4, a slightly greater reduction of ash weight than of total dry, fat-free weight of the bones. Thus, the bones of the animals fed a diet of 4.5% protein lost (or failed to gain in the process of bone reformation) slightly more mineral than they did of organic material. The mean result for percentage ash of the humeri obtained with group 4 is in the direction that would be expected to result from a greater reduction in total ash weight than in dry, fat-free weight, but does not differ statistically from the mean result obtained with the control group.

It has been shown (Armstrong, '46) that the humeri of rats of the strain used in this study continue to grow in length, but at a reduced rate, until an age is reached which is approximately equal to the sacrifice age of the animals used in this study. Thus, the possibility existed that the lower dry, fat-free and ash weights of the humeri of the animals in groups 3 and 4 were due to an interference in bone formation, in the sense of bone growth. The data given in table 3 show that an interference of bone growth is a factor which accounts for a part, but not all, of the differences of the humeri of groups 3 and 4 from those of group 1. While the humeri of groups 3 and 4 were significantly shorter than those of group 1, the lesser lengths of these bones were not in proportion to their lower dry, fat-free and total ash weights. This deduction is made from a comparison of the percentage deviations of the mean results of groups 3 and 4 with those of group 1.

The calcium and phosphorus contents of the ash of the humeri of groups 1 and 4 were not found to be significantly different. The weights of the livers in the 4 groups were closely correlated with their final body weights (averaging 4.79 gm per 100 gm body weight) and sections of this organ from typical animals were normal in histologic structure.

#### SUMMARY AND CONCLUSIONS

The effects on body weight and on the humeri of rats produced by feeding to mature rats for 30 days equal amounts of essentially isocaloric diets containing 4.5, 9.0, 13.5 and 18.0% protein as lactalbumin have been determined. The following conclusions are drawn:

1. A diet containing 4.5% protein will not support body weight of mature rats while diets containing 9.0, 13.5 and 18.0% protein permit an increase in body weight in approximate proportion to the protein content.

2. A diet containing 13.5% protein supports a degree of skeletal constitution in mature rats equal to that of a diet containing 18.0% protein.

3. Diets containing 9.0 and 4.5% protein are inadequate to support normal skeletal constitution in mature rats.

4. Diets containing 9.0 and 4.5% protein interfere with the growth in length of the humeri of rats which are near the end of the period of skeletal growth, but retardation of skeletal growth does not account for all the skeletal changes caused by feeding protein deficient diets.

5. The composition of the bone ash is not affected by feeding protein deficient diets to mature rats.

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# URINARY EXCRETION OF ASCORBIC ACID BY GUINEA PIGS AT DIFFERENT AGES

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TWO FIGURES

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Few studies have been made of the urinary excretion of ascorbic acid in guinea pigs and none have dealt with the question of age. Hess and Benjamin ('34) reported that guinea pigs on an oat diet devoid of ascorbic acid excreted only 0.146 mg each per day, those with a normal intake (carrots and lettuce) excreted 0.3 mg and those with a very high intake (10-60 ml orange juice) excreted only 0.167 mg. No difference was observed between normal animals and those which had moderate scurvy, either as to the initial indophenol-reducing values of the urine or in the values showing the urinary response to ingestion of the vitamin. However, when ascorbic acid was injected intraperitoneally in large amounts similar to the quantities which had been administered orally a sharp rise in urinary excretion occurred, usually on the second or third day after the injections were started. When the injections were stopped, the reduction in urinary concentration of the substance was often quite gradual. Von Euler and Memberg ('35) also failed to find an appreciable excretion of ascorbic acid in the urine of guinea pigs when the vitamin was administered orally.

It is possible that the low values reported by these investigators for urinary excretion of ascorbic acid in guinea pigs on a scorbutogenic diet, or even in normal animals receiving the vitamin orally, may have been due not to the presence of traces of ascorbic acid but to other indophenol-reducing substances.

Zilva ('36) studied the ascorbic acid requirement of the guinea pig and found that an oral dose of 500 mg is necessary for growing guinea pigs (300 gm) before the vitamin is excreted. Von Euler and Memberg ('35), however, had found little excretion with doses as high as 1 gm. In agreement with Hess and Benjamin ('34), Zilva found that urinary excretion is brought about by much smaller doses when the vitamin is injected than when it is administered orally. Ascorbic acid appeared first in the urine when 20 mg was injected. This was the smallest amount which produced the maximum concentration in the tissues. As the dose was increased, the amount excreted rose also.

The problem of the retention of ascorbic acid by humans of different ages has not been given extensive study. In much of the work that has been done thus far, data have been obtained which afford interesting and valuable suggestions but the studies have been concerned with too few subjects and over periods too short to furnish conclusive evidence of an effect of age. An investigation bearing on this problem, which should be specially noted, was conducted by Schuck and Purinton ('43). They followed the daily excretion of ascorbic acid in a group of 63 subjects—52 women and 11 men. They used the saturation test with 500 mg of ascorbic acid administered intravenously. The individual to be tested was given a definite diet, with an ascorbic acid intake of 15 mg daily for the males and 14 mg for the females. After determining the quantity of the vitamin excreted during the first 24 hours after the test dose, Schuck and Purinton calculated the quantity retained by the individual. By subtracting the quantity of ascorbic acid excreted during the test period from the total amount administered, a retention figure was obtained.

Then, by subtracting the value for the ascorbic acid excreted under fasting conditions from the retention figure, a value was obtained which was considered as the quantity of ascorbic acid metabolized and probably therefore required by the individual in question. By this method a metabolism of 113 mg was obtained for the women subjects under 20 years of age, whereas for women between 25 and 50 years of age only 81 mg was metabolized. It is possible that still greater differences might have been found had there been wider differences in age between the various groups, particularly if some were in an active stage of growth. Women between 20 and 25 years of age metabolized slightly more ascorbic acid per unit of body weight than men in the same age group. A somewhat different interpretation of the metabolism data could be made if it were assumed that the subjects may not have been saturated at the beginning of the tests, notwithstanding the fact that they were excreting a small amount of the vitamin during the fasting period. If the younger group, for example, were in a higher degree of unsaturation than the others, the amount metabolized would consist of the average daily amount plus the retention of an additional amount because of the unsaturation. In other words, the vitamin would be used up not only for the day's needs but to make up for the previous deficit as well.

No reports have been made of estimations of ascorbic acid retained by the same individuals at different ages either in man or in animals. Measurements of this kind on humans have not been made up to the present time because of the length of time required and also because of the difficulty of maintaining satisfactory dietary conditions for such extended periods. Data obtained in such investigations would be of considerable interest because of their possible bearing on the ascorbic acid requirement of the individual at different ages. Since guinea pigs pass through the various phases of their life cycle rapidly as compared to humans and since dietary control is a simple matter, they were selected as the experimental subjects for tests of this kind.

## PROCEDURE

Guinea pigs of an inbred strain<sup>1</sup> were placed in metabolism cages at ages ranging from 15 to 26 days and were fed a pelleted scorbutogenic stock diet (commercial) supplemented with 1% of powdered yeast. Fresh food and water were supplied daily. The animals (8 males and 3 females) were weighed daily and were given ascorbic acid intraperitoneally in the proportion of 5 mg per 100 gm of body weight. The solution for injection was prepared by dissolving 15 mg of the vitamin per milliliter of a 0.5% solution of  $\text{NaHCO}_3$ . The animals were kept in 8-inch metabolism cages until 3 months of age, when they were transferred to 10-inch cages. The animal room was maintained at 76 to 80°F.

Five grams of crystalline metaphosphoric acid were placed daily in each bottle for collecting the urine. During the early phases of the experiment this amount appeared to be sufficient for protecting the ascorbic acid against oxidation. Later, as the volume of urine increased, the amount of metaphosphoric acid was also increased. By the time the animals were approximately 4 months old the amount had been raised to 10 gm and this quantity was used for most of the animals for the remainder of the test. For 2 animals which produced an unusually large volume of urine, 12 gm of the acid were used. Control tests were also conducted to determine the ability of the metaphosphoric acid to protect solutions of pure ascorbic acid during a 24-hour period. The amounts of the acid used were found to be adequate under the experimental conditions. Occasional tests were also made with urine added to the ascorbic acid solution.

Determinations of the amount of ascorbic acid excreted in the urine were made daily by the indophenol titration method and approximately every 10 days by the osazone method of Roe and Kuether ('43). The tests were continued by both methods

<sup>1</sup>Animals from family no. 13 were used. This strain was developed by the Genetics Section of the Bureau of Animal Industry of the United States Department of Agriculture. Later, the line was continued by the Genetics Section of the National Cancer Institute, from which the animals here used were obtained.

until the animals reached an age of 6 months and then for 2 months longer by the indophenol method only.

### RESULTS

Per unit of body weight, both methods of assay showed a definite upward trend in excretion with increasing age, leveling off at about the time growth was nearing completion. Figure 1 shows the curves for male animals at successive ages, the

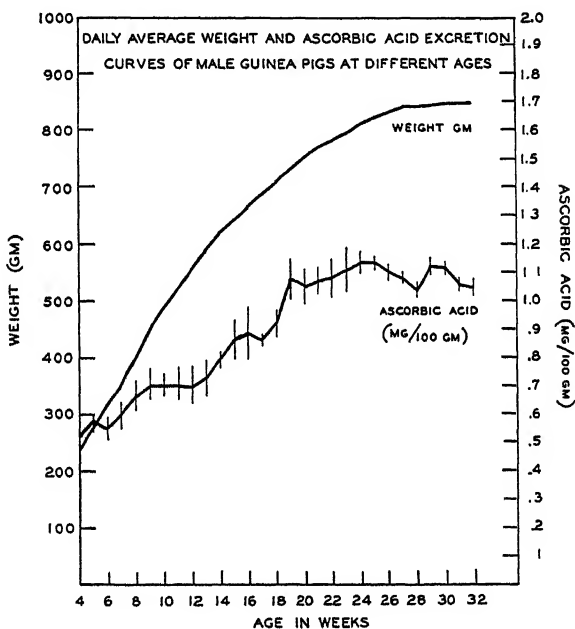


Fig. 1 Daily average weight and ascorbic acid excretion curves of 8 male guinea pigs at different ages.

average values for weight being expressed in grams, and those for excretion of ascorbic acid as milligrams per 100 gm of body weight. The standard deviations for the excretion values obtained with the indophenol method are also shown. Because of the infrequency of the determinations by the osazone method, the excretion values yielded by this technique have not been plotted. In general, there was fair agreement

in the results obtained by the 2 methods. There were not enough female animals to permit construction of growth and excretion curves comparable to those shown for the male animals. The excretion values of the females tended to be more fluctuating than those of the males, and after sexual maturity they were definitely lower than those of the male animals. Of the total ascorbic acid administered, 10.6% was excreted

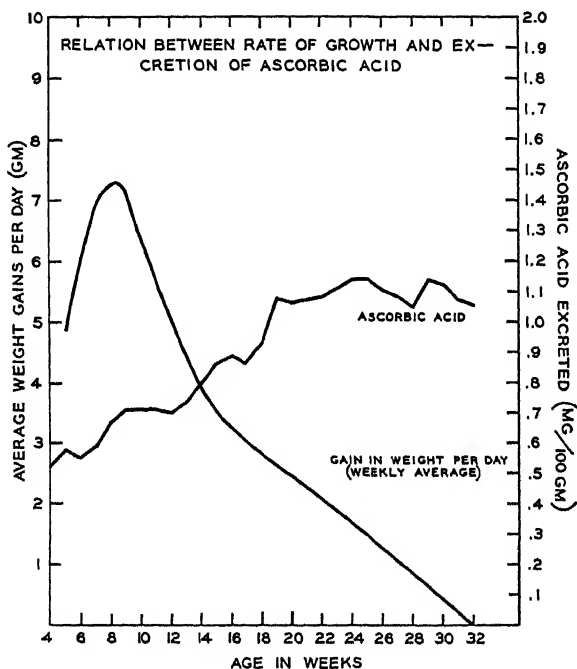


Fig. 2 Relation between rate of growth and excretion of ascorbic acid.

in the urine at the age of 6 weeks, 13% at 9 weeks, and 23% at 24 weeks of age. Figure 2 shows the relation between the rate of growth of male guinea pigs and the excretion of ascorbic acid per unit of weight. The period of most rapid gain in weight in both sexes, that is, up to the twelfth or thirteenth week (the maximum gains occurring during the eighth and ninth weeks) is the time of lowest rate of excretion of the vitamin per unit of body weight. There is an inverse relation

between the general direction of the 2 curves, the excretion per unit of body weight being, in general, lowest when the weight-rate gains are highest. As the rate of growth diminishes, the excretion rises.<sup>2</sup> The increase in excretion per unit of body weight continues for the male animals until the twenty-fourth week and for the females until around the twenty-first week. From the fourteenth to the nineteenth week the rate of growth falls off only moderately whereas ascorbic acid excretion per unit of weight rises markedly.

#### DISCUSSION

There was considerable variation throughout the entire experimental period in the excretion of ascorbic acid from day to day. If the values were high 1 day, they tended to be lower the next. The weekly averages thus give a better indication of the excretion than the values for single days.

Both the excretion and the growth for the period extending from the third to the ninth week require further study. In another test it would be desirable to begin the injections shortly after birth and also to use a higher dosage so that when the excretion and growth studies are begun at the time of weaning the animals would be saturated, or nearly so. It is probable, however, that at this age a single injection a day even with a relatively high dosage would be insufficient to maintain a saturated condition throughout a 24-hour period. It is also possible that with the animals saturated, or nearly so, the excretion curve would be practically flat from weaning time to the fourteenth week instead of showing the rise here found from the fifth to the ninth or tenth week. It is also possible that, with conditions permitting the maintenance of a favorably high blood level of ascorbic acid throughout each 24-hour period, the growth curve would show a plateau at a high

<sup>2</sup> In other tests it has been found that with respect to the retention of ascorbic acid (Reid, '47a), mature animals which are required to build new tissues in the healing of wounds behave apparently like younger animals during the healing period.



level during this early period instead of a rise in rate to a peak at the seventh week.

The present results have shown a difference in excretion of approximately 0.4 to 0.5 mg/100 gm between the 8 week-old and the adult animal 6 months old. Presumably this may represent an amount of the vitamin actually used up daily by 100 gm of tissue during the growing period. For a 300-gm animal injected intraperitoneally, there would thus be a daily utilization of 1.2 to 1.5 mg. This is approximately the daily requirement of the vitamin as reported by Dann and Cowgill ('35). They found that with oral administration 1 ml of lemon juice (0.4–0.6 mg ascorbic acid) per 100 gm of body weight and irrespective of age is required to protect guinea pigs from scurvy, as measured by the Höjer tooth method.

Results of the present tests suggest strongly, though they do not prove, that the ascorbic acid requirement of the young animal is greater than that of the adult. The possible discrepancy between these results and those of Dann and Cowgill may be caused by the difference in mode of administration of the vitamin.

Results obtained in later studies (Reid, '47b) suggest that the major portion of the intraperitoneally injected vitamin is destroyed in the contents of the digestive tract. It appears possible, however, that the 10% greater loss in the growing animal at 9 weeks as compared to that in the adult animal at 6 to 8 months may constitute a fraction disappearing in a different way. This 10% fraction may conceivably be used up in the body tissues in growth. The fact that the excess amount which disappears during growth is the same as the amount known to be required by the young animal lends some support to this hypothesis. However, the possibility of the loss occurring in other ways is recognized.

#### SUMMARY

A study has been made of the retention of ascorbic acid by guinea pigs at different stages of growth. The animals were fed a scorbutogenic diet supplemented daily with as-

corbic acid injected intraperitoneally in relation to body weight.

The results of daily determinations of the ascorbic acid excreted in the urine, per unit of body weight, show a definite upward trend with increasing age, leveling off at about the time growth is nearing completion. The excretion at 8 weeks of age was 0.67 mg/100 gm and at 24 weeks, 1.14 mg. Of the total ascorbic acid administered, 10.6% was excreted in the urine at the age of 6 weeks, 13% at 9 weeks, and 23% at 24 weeks.

An inverse relation was observed between the excretion of ascorbic acid per unit of body weight and the rate of growth at different ages.

The results show that a greater amount of ascorbic acid disappears during the period of rapid growth than during adulthood. Further study is necessary before conclusions can be drawn as to an effect of age on the ascorbic acid requirements of the guinea pig.

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# THE VITAMIN A REQUIREMENT FOR GROWTH OF FOXES AND MINKS AS INFLUENCED BY ASCORBIC ACID AND POTATOES

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Smith ('42) first described the symptoms of vitamin A deficiency in silver foxes. From limited tests he observed that 15 to 25 I.U. of vitamin A per kilogram of body weight daily were necessary to prevent deficiency. There was no appreciable storage, in the liver, of vitamin A until approximately 4 times this amount was fed. Bassett et al. ('46a) extended these studies to determine the vitamin A requirement under actual ranch conditions. They occasionally observed deficiency symptoms on intakes as high as 100 I.U. of vitamin A per kilogram of body weight daily when these pups were drastically depleted of vitamin A before they were weaned. In these experiments the diets were extremely low in carotene or vitamin A and contained some ingredients not commonly used in feeding foxes. It seemed desirable to determine whether or not actual deficiency would be experienced when more conventional rations were fed. As no previous work had been carried on to determine the vitamin A requirements of mink, it was decided to conduct the same type of experiment with that species also.

Mathiesen ('42) reported that foxes do not require a dietary source of ascorbic acid. Since most fur farmers feed vegetables which contain ascorbic acid, however, it seemed important to test its value in the diet.

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## EXPERIMENTAL

Eighty silver fox pups and 60 mink kits, divided equally according to sex, were allotted by chance into 10 groups. The foxes were 50 and the minks 64 days of age when started on experiment. The percentage composition of the basal diet was as follows: ground horsemeat 10.0, soybean oil meal 3.5, beef meal 2.9, skim milk powder 0.6, rolled oats 5.8, bread crumbs 5.6, cookie crumbs 11.2, brewers' dried yeast 4.7, wheat germ meal 5.8, ground limestone 1.3, and water 48.6. This diet contained on analysis: protein 28.8%, ether extract 6.4%, crude fiber 2.5%, nitrogen free extract 56.4%, and ash 5.9% on a dry matter basis, and only traces of carotene. The basal diet was supplemented with vitamin A for different groups of animals at the rates of 0, 50, 500, 2500 and 5000 I.U. of vitamin A per pound of moist, mixed feed. Five other groups receiving the above amounts of vitamin A were given, in addition, 20 mg of ascorbic acid per pound of feed to test whether (1) this vitamin would influence the vitamin A requirement of foxes and minks, and (2) vitamin C would affect their growth rates. The foxes were fed once daily, the minks twice, and records were kept of the feed consumed. All animals were weighed at the start of the experiment, and the foxes were weighed each 2 weeks, and the minks each week thereafter. An average of 2 weights taken on successive days constituted the initial and final weights. Weekly observations were made to detect the onset of any deficiency symptoms. Animals were killed when their pelts were prime, and observations made on fur growth and quality and on the general condition of the animals.

Blood samples obtained by cardiac puncture were cooled and shipped by parcel post in the late afternoon following collection to Cornell University, Ithaca, N. Y. Immediately after their arrival the following day, the samples were centrifuged and analyzed for carotene and vitamin A. It is possible that slight losses of vitamin A occurred from this handling, although tests have shown that vitamin A is not appreciably reduced when plasma or even whole blood is stored at approx-

imately 35°F. for at least a week (Loosli, '47). Livers collected from all minks and from at least 5 foxes in each lot were ground and a composite sample frozen and stored 6 to 7 weeks before vitamin A analysis was made.

Comparative pelt quality was determined by experts of a New York fur auction company.

## RESULTS

### *Studies with foxes*

There were no appreciable differences in rates of growth on the various intakes of vitamin A or ascorbic acid. Total food intake appeared to be slightly greater when the diet contained 500 I.U. or more of vitamin A per pound, but no ascorbic acid, than lower levels. The quality of the pelts of foxes appeared to be slightly less desirable on low intakes of vitamin A unless ascorbic acid was fed, but the number of animals studied was too small to show a significant trend.

Symptoms of vitamin A deficiency (i.e., head cocking, whirling, and weaving or staggering gait) were seen in 38% of the foxes receiving no extra vitamin A or ascorbic acid, while the addition of 20 mg of ascorbic acid per pound of feed prevented the occurrence of symptoms.

That there may be an interrelationship between vitamin A and ascorbic acid in the nutrition of foxes is further supported by the vitamin A analyses of blood serum and livers (table 1).

There appears to be a higher level of vitamin A in the blood serum and livers on the vitamin-A free diets with ascorbic acid than on the diet containing neither of these vitamins. There was a consistent increase in the vitamin A content of the blood serum and livers of foxes as the dietary intake rose.

### *Studies with minks*

With the possible exception of the 0 vitamin A level, there were no differences in gain in weight which can be correlated with the amount of vitamin A or with the omission of ascorbic acid in the diet. At the 0 vitamin A level the average gain in

weight was 0.20 pound less than the average of all other levels tested, and 0.38 pound less than the value for normal minks of the same strain. This difference may have been due to the low vitamin A intake. Differences in food consumption did not appear to be related to the intakes of vitamin A or ascorbic acid. Vitamin A deficiency symptoms, such as occur in foxes and other animals, were not observed. This study suggests that minks require less vitamin A per pound of feed

TABLE 1

*The vitamin A content of the serum and livers of foxes and minks as affected by amount of the vitamin in the feed.*

ASCORBIC ACID OF FEED	VITAMIN A PER POUND OF FEED (I.U.)				
	0	50	500	2500	5000
mg/lb.	Vitamin A content of blood serum of foxes ( $\mu\text{g}/\text{ml}$ )				
0	$0.28 \pm .01^1$	$0.43 \pm .03$	$1.11 \pm .06$	$3.35 \pm .37$	$5.66 \pm .82$
20	$0.63 \pm .16$	$0.39 \pm .03$	$1.30 \pm .28$	$2.21 \pm .19$	$5.40 \pm .22$
	Vitamin A content of fox livers ( $\mu\text{g}/\text{gm}$ ) <sup>2</sup>				
0	0.15	0.25	0.55	2.86	3.73
20	0.21	0.30	0.62	1.34	3.77
	Vitamin A content of blood serum of minks ( $\mu\text{g}/\text{ml}$ )				
0	$0.22 \pm .01$	$0.19 \pm .02$	$0.23 \pm .04$	$0.78 \pm .02$	$2.64 \pm .75$
20	$0.31 \pm .14$	$0.46 \pm .12$	$0.33 \pm .18$	$1.02 \pm .16$	$1.46 \pm .12$
	Vitamin A content of mink livers ( $\mu\text{g}/\text{gm}$ ) <sup>2</sup>				
0	0.00	2.06	2.06	18.45	72.10
20	2.06	2.57	2.64	14.60	202.00

<sup>1</sup> Mean and standard error of the mean.

<sup>2</sup> Analyses of composite liver samples.

than foxes, or that their liver reserves at the start of the study (64 days of age) were adequate to carry the control groups until pelting. Death losses among animals that did not receive ascorbic acid were 50, 17, 33, 0 and 33%, respectively, for the 5 levels of vitamin A, in ascending order. Corresponding figures for the animals that were given ascorbic acid were 50, 0, 33, 67 and 17%. Post-mortem examination revealed hemorrhagic areas in the region of the heart and lungs, over

the skull, and throughout the abdomen and intestines. The stomach and intestines frequently contained blood, and in some instances the bladder was enlarged and filled with bloody urine. Emaciation and a severe anemia, indicated by the pale color of the liver, spleen, kidneys, heart and blood, coupled with a blue and transparent appearance of the intestines, were common. A similar pathology has been described by Kennedy ('46).

Examination of the pelts revealed no certain trend in quality related to the intake of vitamin A.

The analyses of blood serum and livers for vitamin A (table 1) show a generally consistent increase in the vitamin A content as the intake of the vitamin rose. In most cases there was also a higher content of vitamin A when ascorbic acid was fed; this was observed also with foxes.

#### *Effects of feeding potatoes to foxes and minks*

Potatoes as 50% of the diet were fed to 42 foxes and 48 minks. The percentage composition of the diet was ground meat 10.0, soybean oil meal 3.0, beef meal 2.5, dried skim milk 0.5, oatmeal 5.0, dried brewers' yeast 3.9, irradiated yeast 0.1, wheat germ meal 5.0, cooked potatoes 50.0, and water 20.0. On analysis, the dry matter was found to contain 33.4% protein, 3.7% ether extract, 3.6% crude fiber, 53.8% nitrogen free extract, and 5.5% ash. Half of each group were given a supplement of 5000 I.U. of vitamin A per pound of mixed feed. Data collected are shown in table 2.

#### DISCUSSION

Foxes that received 5000 I.U. of vitamin A per pound of feed gained an average of 1.05 pounds more than those receiving no supplement. Conversely, minks that received no vitamin A supplement gained an average of 0.18 pound more than those furnished the extra vitamin, largely because of a greater accumulation of fat. Of the minks that were not given additional vitamin A, 79% were fat or extra fat, but only 55%



of those fed the vitamin supplement were considered fat. Some foxes fed the 50% potato diets consumed as much as 2.0 pounds of feed a day. Ordinarily a growing fox does not consume more than 1.25 pounds of feed per day. Those that received 5000 I.U. of vitamin A per pound of feed consumed 35%, and those furnished no supplement 21%, more food than an average of 80 foxes fed a diet that did not include potatoes. Mortality was greater for both species in groups that did not receive extra vitamin A. The content of vitamin A in the blood serum and livers of both foxes and minks fed

TABLE 2

*The mortality rate, gain in weight, food intake and vitamin A content of serum and livers of foxes and minks receiving diets containing 50% potatoes with and without added vitamin A.*

VITAMIN A PER POUND OF FEED	MORTALITY	AVERAGE GAIN IN WEIGHT	AVERAGE FOOD INTAKE	VITAMIN A CONTENT	
				Serum	Liver
I.U.	%	lbs.	lbs.	$\mu\text{g/ml}$	$\mu\text{g/gm}$
Foxes					
0	14	8.58	239.3	$0.92 \pm .09$	0.45
5000	0	9.63	279.0	$6.45 \pm .52$	6.07
Minks					
0	21	1.35	79.8	$0.32 \pm .08$	23.40
5000	8	1.17	76.7	$3.64 \pm .28$	363.00

potatoes was higher than for comparable animals not fed potatoes. Because vitamin A was added per unit of diet, the intake increased with the greater food consumption. It is not clear whether this would fully explain the higher serum and liver contents, or whether the potatoes may have enhanced the utilization or storage of the vitamin. The influence of ascorbic acid and of potatoes in increasing supplies of vitamin A in the serum and liver is clearly shown in table 3. The effect here observed appears to be similar to the sparing action of ascorbic acid when fed with low intakes of carotene (Hickman et al., '44). A relationship between vitamins A and C in calf nutrition was reported by Boyer et al. ('42).

The magnitude of some of the increased stores of vitamin A and the influence, for all species, of antioxidants and other dietary ingredients on the conservation and utilization of this vitamin emphasize the need for extension of these studies.

TABLE 3

*The influence of ascorbic acid and potatoes on the vitamin A content of the serum and liver.*

SUPPLEMENT	AVERAGE VITAMIN A CONTENT WHEN —			
	No vitamin A fed		5000 I.U. vitamin A fed per pound of diet	
	Serum	Liver	Serum	Liver
	$\mu\text{g/ml}$	$\mu\text{g/gm}$	$\mu\text{g/ml}$	$\mu\text{g/gm}$
Foxes				
Basal only	0.28	0.15	5.66	3.73
Basal + ascorbic acid	0.63	0.21	6.40	3.77
Basal + potatoes	0.92	0.45	6.45	6.07
Minks				
Basal only	0.22	0.00	2.64	72.10
Basal + ascorbic acid	0.31	2.06	1.46	202.00
Basal + potatoes	0.32	23.40	3.64	363.00

TABLE 4

*Influence of the vitamin A intake upon the serum and liver content of growing foxes.*

VIT. A INTAKE/ KG BODY WT.	NO. OF FOXES	AVERAGE VIT. A CONTENT		VIT. A INTAKE/ KG BODY WT.	NO. OF FOXES	AVERAGE VIT. A CONTENT	
		Blood serum	Liver			Blood serum	Liver
I.U.		$\mu\text{g/ml}$	$\mu\text{g/gm}$	I.U.		$\mu\text{g/ml}$	$\mu\text{g/gm}$
0	30	0.00	0.00	200	6	1.98	1.20
15	19	0.00	0.00	600	6	3.43	1.61
20	10	0.00	0.00	830	16	2.78	2.10
25	24	0.32	0.25	1825	16	6.03	3.75
50	24	0.40	0.26	2500 <sup>1</sup>	21	6.45	6.07
100	16	0.55	0.40	5000	3	0.81	6.13

<sup>1</sup> Diet contained 50% potatoes.

The available data for foxes (Smith, '42; Bassett et al., '43, '46a) showing the vitamin A intake and corresponding blood and liver contents are summarized in table 4. These data

show that a vitamin A intake of 25 I.U. per kilo of body weight gave measurable amounts in the blood and liver, whereas these tissues were practically devoid of vitamin A on an intake of 20 I.U. Moore et al. ('43) found that in calves 15  $\mu$ g of vitamin A per 100 ml plasma are necessary to prevent deficiency symptoms. Thus, using this criterion, 25 I.U. per kilo of body weight (11.4 I.U. per pound of weight, or 2.8  $\mu$ g), which is approximately equivalent to 10 I.U. per pound of moist diet, may be considered the minimum requirement for the growth of foxes. This minimum requirement (6.2  $\mu$ g—assuming 1  $\mu$ g = 4 I.U.) is the same as that found for rats and farm animals by Guilbert et al. ('40), and supports their hypothesis that the vitamin A requirement is related to body weight and that it is approximately the same for all mammals. Bassett et al. ('46b) have also shown that foxes utilize carotene as a source of vitamin A. The quantitative requirement, while not yet accurately defined, is not out of line with the predicted need based on studies with other animals.

A higher apparent requirement of vitamin A to prevent the occurrence of deficiency symptoms reported by Bassett et al. ('46a) appears to have been the result of severe depletion of the pups before they were started on the diets containing the vitamin A supplements.

The present study further emphasizes the view that both "growing" and "pelting" diets fed to young foxes and minks, which usually include vegetables and fresh liver, contain sufficient vitamin A, and therefore the addition of this vitamin or of carotene concentrates is unnecessary.

#### SUMMARY AND CONCLUSIONS

Eighty silver foxes and 60 mink kits were allotted to experimental groups receiving a basal diet low in vitamin A and supplemented with 0, 50, 500, 2500 and 5000 I.U. of vitamin A per pound of feed. Half of each of the above groups received 20 mg of ascorbic acid per pound of feed. Blood samples, taken by cardiac puncture, and livers were collected and assayed for vitamin A.

Gross vitamin deficiency symptoms appeared among foxes on the unsupplemented basal ration, but diets containing 50 I.U. or more of added vitamin A per pound were satisfactory for growth, prevented deficiency symptoms, and provided a vitamin A content in the blood and liver that rose consistently as the intake increased. The addition to the basal diet of 20 mg of ascorbic acid per pound of feed apparently prevented the appearance of vitamin A deficiency symptoms and increased slightly the vitamin A content of the blood serum and liver.

The content of vitamin A in the blood serum and livers of minks also increased consistently with the intake of the vitamin, and was greater when ascorbic acid was added to the basal diet. Other differences could not be correlated with either vitamin A or ascorbic acid.

Results indicate that an interrelationship exists between vitamin A and ascorbic acid in the nutrition of foxes and minks.

A diet containing 50% cooked potatoes was fed to 42 silver foxes and 48 minks. Food intakes and the content of vitamin A in the blood serum and liver were higher than for comparable animals not fed potatoes.

The minimum vitamin A requirement necessary to provide satisfactory growth and a detectable content of vitamin A in the blood serum and liver of foxes is approximately 25 I.U. per kilogram of body weight per day. This is equivalent to 6.2  $\mu$ g per kilo (11.4 I.U., or 2.8  $\mu$ g per pound of body weight), which can be supplied by adding approximately 10 I.U. of vitamin A per pound of the moist diet.

Under ordinary ranch conditions where little or no vitamin A depletion occurs among fox pups during the suckling period, diets of the type fed by most ranchers contain sufficient vitamin A so that no supplementation is necessary. The inclusion of adequate amounts of leafy vegetables, tomatoes, apples, or a substantial quantity of cooked potatoes in the diet would preclude the necessity for vitamin A supplementation.

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# COMPARATIVE GROWTH-PROMOTING VALUES OF THE PROTEINS OF CEREAL GRAINS

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Close to one-third of the total protein in the human dietary in the United States is supplied by cereals, chiefly wheat, corn, oats, rye, barley and rice. Cereal grains have been from antiquity an important source of protein in the human diet.

Numerous studies have been conducted by feeding experiments over a period of many years on the comparative nutritive value of the proteins of cereal grains. Recorded results of much of the earlier work, however, are confusing and conflicting. Not infrequently it was concluded that the grains differed little, if any, among each other, with respect to their protein nutritional properties, and that they were all of rather low value. These investigations were conducted in different laboratories, under various conditions, and in most cases on only a few of the grains at the same time. Because of lack of uniformity in experimental procedures, variations in the compositions of basal diets and inadequacy of vitamin and mineral supplements, the results do not afford a satisfactory basis for comparing the cereal grains with respect to the nutritive value of their proteins.

Several of the early investigators on the protein nutritional value of cereal grains concluded that rye, wheat, corn, barley and oats vary little with respect to the nutritive value of their proteins (Sherman and Winters, '18; McCollum et al., '19;

Osborne and Mendel, '20; McCollum and Simmonds, '25; Johnson and Palmer, '34).

Recorded data on the nutritive value of barley protein are few, and the work was done many years ago. The values observed are conflicting and they do not afford a satisfactory basis for comparison with those of other grains. Steenbock et al. ('18) and McCollum et al. ('19) found comparatively low values for barley protein. Working with the entire ground seed, Osborne and Mendel ('20) stated that the results "leave no doubt of the adequacy of the barley proteins as a whole in the nutrition of growth." With pearled barley, however, the animals (rats) grew very slowly.

Corn has been generally regarded as an inadequate source of protein for growing animals. Zein, constituting from 41 to 52% of the protein of corn, is well-known to contain little or no tryptophane and to be low in lysine, and these amino acids are not satisfactorily compensated by the other proteins present. Maynard, Fronda and Chen ('23) found corn to have a low protein efficiency. Grau ('46) showed that corn gluten meal had a low growth value for chicks and that it required addition of arginine, lysine and tryptophane. Boas-Fixsen et al. ('34), in nitrogen equilibrium and growth studies, concluded that only a slight difference exists between the nutritive value of the proteins of whole wheat and whole maize for support of growth in the young rat.

Compared with most cereal grains, rye has received little attention with regard to its protein value. Osborne and Mendel ('20) reported that the efficiency of rye protein is comparable with that of other grains studied. Later, however, in re-evaluating Osborne and Mendel's data, Mitchell and Hamilton ('29) stated that the superiority of rye protein over wheat is clearly demonstrated. McCollum et al. ('19) observed that rats grew at only approximately half the normal rate. In feeding experiments with rye and wheat breads, Kon and Markuze ('31) obtained results indicating the superiority of the rye breads with respect to the biological value of the protein. Johnson and Palmer ('34) concluded that rye protein

is comparable with that of wheat, corn and oats, and that lysine, but not tryptophane, is the first limiting factor.

Considerable confusion exists in the earlier publications regarding the nutritive value of the protein of oats. Conflicting statements may be found by the same authors at different times, as pointed out by Osborne and Mendel ('20). In general, however, the conclusions point to a higher value for the protein of oats than for most of the other cereal proteins. In nitrogen balance studies with human adults, Sherman et al. ('19) found that the protein of oats and maize had about equal value. By means of nitrogen metabolism experiments with rats, Mitchell ('24) showed a distinct superiority of oat protein over corn protein at different levels of protein intake. Hoagland and Snider ('27) noted a close similarity between the protein of wheat and oatmeal. Hartwell ('26) found that the protein of oats was of good nutritive value, although the diet was supplemented only with mineral salts and butter. Mitchell and Smuts ('32) showed that addition of lysine to an oat diet resulted in a distinct though slight increase in the growth of rats. The protein of rolled oats was found by Mitchell and Block ('46) definitely superior to that of other cereals tested, whether processed or unprocessed.

That rice does not show the marked differences which other cereal proteins do in respect to their amino acid composition was demonstrated by Osborne et al. ('15). They pointed out that rice protein yields a relatively large amount of each of the basic amino acids, arginine, histidine and lysine. Later, Osborne and Mendel ('18) emphasized the superior growth-promoting value of the protein of rice compared with that of corn. McCollum and Simmonds ('17) found rice protein comparable with that of wheat and corn. In metabolism experiments with rats, Mitchell ('24) demonstrated that rice protein had a higher biological value than that of corn and oats. Li ('30) found rice superior to barley. Recently, Sure ('46, '47) demonstrated a marked superiority in growth-promoting value and in protein efficiency of polished rice over that of wheat flour. Mitchell and Block ('46) reported



that lysine is the limiting amino acid in rice protein. In feeding experiments with rats, Kik ('39) found that polished rice and brown rice have essentially the same growth value, but that brown rice protein is better utilized, having a biological value of 72.7 compared with 66.6 for polished rice. Kik ('40) also found that cystine, methionine and lysine supplement the proteins of whole and of polished rice to a slight extent. Addition of tryptophane did not confer any beneficial effect.

#### EXPERIMENTAL

The cereal products used, with the exception of 2 samples of hard, red spring wheat,<sup>1</sup> were purchased locally in the open market. Their protein contents are given in table 1.

TABLE 1

*Protein contents of the cereal products.  
The percentages are calculated on an air-dry basis.<sup>1</sup>*

MATERIAL	PROTEIN	MATERIAL	PROTEIN
	%		%
Barley, pearled .....	9.15	Rice, polished .....	5.24
Corn, whole yellow .. .	9.19	Rice, brown .....	9.04
Oats, rolled .. .	14.87	Wheat, hard, spring ....	14.24
Rye, whole .	10.96	Wheat, soft, winter	11.02

<sup>1</sup> The following conversion factors were used in calculating the amounts of protein in the materials from their nitrogen content: Wheat, rye, barley and oats, 5.83; corn, 6.25; rice, 5.95 (Jones, '31).

The growth-promoting values of the proteins were determined by feeding experiments with young albino rats. The materials, ground to a fine meal, served as the sole source of protein in diets supplied with the essential non-protein dietary factors. As far as their N contents permitted, the test substances were incorporated in the diets in quantities to supply protein at 4.5, 7.5, 9.5 and 12% levels. Because of their

<sup>1</sup> One of the hard, red spring wheat samples was obtained from the Agricultural Station, Dickenson, North Dakota. The other sample was a Montana wheat supplied through the kind offices of Dr. F. L. Dunlap, of the Wallace Tiernan Company, Chicago, Illinois.

low N contents, not all of the materials could be used at the higher levels without displacing some of the essential accessories. Polished rice, for instance, could be introduced into the diet at only the 4.5% protein level. Only hard wheat and rolled oats permitted comparison at the 12% protein level. The average net gain in weight of each group of animals over a period of 6 weeks and the average gain in weight per gram of protein consumed were used as standards of comparison for evaluating the proteins of the materials studied.

All diets contained 2% cod liver oil and 2% salt mixture (Hubbell, Mendel and Wakeman, '37). The remainder of each of the diets consisted of one of the protein sources listed in table 1, corn oil 8%, and sufficient dextrinized cornstarch to adjust the protein content to the desired levels. A vitamin mixture was incorporated in the dextrinized starch which provided the following constituents in milligrams per 100 gm of diet: thiamine hydrochloride, 0.2; pyridoxine hydrochloride, 0.2; riboflavin, 0.3; calcium pantothenate, 0.3; niacin, 1; and choline hydrochloride, 10. These accessories, in aqueous-alcoholic solution, were added to the dextrinized starch and the mixture was dried at 50°C. to the original weight of the starch.

The data presented represent closely-agreeing results obtained in 2 sets of experiments conducted 1 year apart in which were employed 21 lots of albino rats comparable in all respects. Most of the lots consisted of 20 animals each, equally divided with respect to sex and having initial weights of 45 to 60 gm. They were housed in individual cages having wide mesh screen bottoms and kept in an air-conditioned room maintained at 76° ( $\pm$  2°)F. The animals were weighed and fed ad libitum twice weekly. Fresh diets were prepared in kilogram quantities each week.

#### RESULTS AND DISCUSSION

The average weight gains of the animals are given in table 2. The nutritive values of the proteins of the grains, compared both with respect to body weight gains and gains

per gram of protein consumed, were found in general to rank in the same order when determined by both procedures.

The order of values of some of the grains as compared with each other varied somewhat with the different protein levels at which they were incorporated in the diets. Oats, rice and rye, however, at each of the protein levels at which they were used, proved superior to all of the other grains.

TABLE 2

*Comparative growth-promoting value of the proteins of several cereal grains fed as the sole source of protein in the diet. (Tests conducted by the ad libitum method of feeding over a period of 6 weeks.)*

MATERIAL	AVERAGE WEIGHT GAINS	AVERAGE GAIN/GM PROTEIN CON- SUMED	AVERAGE FOOD CON- SUMED	AVERAGE WEIGHT GAINS	AVERAGE GAIN/GM PROTEIN CON- SUMED	AVERAGE FOOD CON- SUMED
	gm	gm	gm	gm	gm	gm
4.5% protein in diet				7.5% protein in diet		
Corn, yellow	18 ± 2.49	1.42	279	40 ± 2.49	1.56	367
Wheat, hard	18 ± 0.94	1.72	234	43 ± 2.11	1.55	362
Wheat, soft	20 ± 1.32	1.74	216	28 ± 1.68	1.21	326
Barley, pearled	19 ± 1.89	1.55	266	39 ± 2.22	1.67	324
Rice, brown	24 ± 1.50	1.92	288	74 ± 2.30	2.31	418
Rice, polished	33 ± 2.25	2.22	339	..	..	..
Rye	35 ± 2.40	2.26	337	60 ± 2.29	2.16	381
Oats, rolled	38 ± 2.10	2.23	369	59 ± 2.47	2.12	358
9.5% protein in diet				12% protein in diet		
Wheat, hard	68 ± 2.65	1.60	444	76 ± 2.70	1.47	369
Wheat, soft	66 ± 2.35	1.68	414	..	..	..
Rye	74 ± 2.44	1.83	434	..	..	..
Oats, rolled	99 ± 2.76	2.48	414	118 ± 5.64	2.25	428

<sup>1</sup> Including the probable error of the mean result calculated according to the formula  $\sqrt{2d^2/n} - 1/\sqrt{n} \times 0.6745$ , where "d" is the deviation from the mean and "n" is the number of observations (Sherman, '37).

No significant difference was observed between hard and soft wheat, except at the 7.5% protein level. At this level the hard wheat diet supported an average weight gain of 43 gm as compared with 28 gm for the soft wheat. Statistical analysis of the data shows that this difference is significant. The ratio of the mean difference in growth to the probable error of

the difference is 5.5. This implies that the chances are more than 1,300 to 1 that the difference was not fortuitous.

At the 4.5% protein level, corn, hard wheat, soft wheat, barley and brown rice showed essentially the same values. Statistically significant greater gains, however, were made on the oats, rye and white rice diets. At this level white rice showed a significantly higher value than brown rice, and about equaled rye. At the 7.5% level brown rice surpassed rye and oats by odds of 142 to 1. Oats and rye showed values significantly higher than those for hard wheat, barley and corn.

Although at the 9.5% protein level the animals fed rye showed somewhat greater gains than did those that received hard and soft wheat, the differences are not statistically significant.

The superiority of oats, compared with hard and soft wheat at the 9.5% protein level, proved to be highly significant. The oat diet at this level supported an average weight gain of 99 gm as compared with 74 gm for rye, the difference representing odds greater than 100,000 to 1 in favor of oats.

The animals receiving the oat diet at 12% protein level showed an average weight gain of 118 gm as compared with 76 gm gained by those on the hard wheat regime. A statistical analysis of the data shows that the ratio of the mean difference in weight gain to the probable error of the difference is  $\pm 6.7$ . With  $\pm 6E$  the chances are 19,200 to 1 that another random observation or the mean of any equal random sample will fall within the range  $6E$ .

It is unfortunate that all of the cereals could not have been compared at the higher protein levels. It has been our experience that, in general, optimum growth of young rats fed with plant protein foods is attained at 12 to 15% protein levels in the diet, and that satisfactory comparisons can be made when the material is fed at 10 to 12% levels. Some of the variations in the order of values found when the materials were fed at the low protein levels of 4.5 and 7.5% as compared at the higher levels can well be attributed to borderline

deficiencies in one or more essential amino acids which were supplied in large quantities at the higher protein levels.

A compilation of the amino acid contents of the cereal grains investigated is given in table 3. A strict correlation between amino acid composition and growth-promoting values of the cereals used at the different protein levels cannot be satisfactorily made. Several factors may be involved besides the percentages of amino acid present, such as possible interrelationships between both essential and non-essential amino

TABLE 3<sup>1</sup>  
*Percentages of amino acids calculated to 16% nitrogen.*

	ROLLED OATS	RYE	RICE, WHITE	WHEAT	BARLEY	CORN
Nitrogen	2.73	1.98	1.26	3.07	1.86	2.22
Arginine	7.4	5.4	8.7	4.5	4.5	4.7
Histidine	2.2	2.2	2.3	2.0	1.8	2.2
Lysine	3.0	3.3	2.8	2.5	2.4	2.3
Tryptophane	1.3 <sup>2</sup>	1.3 <sup>3</sup>	1.3 <sup>2</sup>	1.4 <sup>3</sup>	1.1 <sup>4</sup>	0.5 <sup>4</sup>
Leucine	6.5 <sup>4</sup>	6.2 <sup>3</sup>	7.7 <sup>2</sup>	6.8 <sup>3</sup>	5.5 <sup>4</sup>	15.0 <sup>4</sup>
Isoleucine	4.2 <sup>4</sup>	4.0 <sup>3</sup>	5.1 <sup>2</sup>	3.6 <sup>3</sup>	3.8 <sup>4</sup>	6.4 <sup>4</sup>
Valine	5.3	5.0	6.3	4.1	5.1	5.3
Phenylalanine	4.6	3.0	4.6	3.8	5.7	4.8
Threonine	3.6	3.9	3.6	3.0	3.6	3.9
Methionine	1.0	1.1	1.4	1.0	1.0	1.4

<sup>1</sup> With the exceptions indicated, the above values were determined microbiologically in this laboratory by Dr. M. J. Horn and Mr. A. Blum.

<sup>2</sup> Block and Bolling ('45).

<sup>3</sup> Stokes et al. ('45).

<sup>4</sup> Baumgarten et al. ('46).

acids, and the varying threshold limits of amino acids at the different protein levels at which the materials were fed. Mitchell and Block ('46) have pointed out that the amino acid make-up of a protein may not be the only considerable factor limiting its utilization within the animal body. The figures given in the table indicate that oats, rice and rye excel with respect to their contents of lysine and arginine, and that they are surpassed in isoleucine only by corn. Rice, containing the highest percentage of arginine and valine, leads also, with

the exception of corn, in leucine and isoleucine. Corn is characterized by its low tryptophane content and high values for leucine and isoleucine. All of the cereals studied show marked similarity with respect to their histidine and methionine values; and, with the exception of corn, with respect to tryptophane as well.

#### SUMMARY

The growth-promoting values of the proteins of whole yellow corn, hard and soft wheat, pearled barley, brown and polished rice, rye and rolled oats were compared by means of ad libitum feeding experiments with young albino rats. As far as their N contents permitted, the materials were incorporated at 4.5, 7.5, 9.5 and 12% protein levels in diets approximately isocaloric and nutritionally adequate with respect to dietary factors other than protein.

The results were evaluated on the basis of weight increases and gains per gram of protein consumed over 6-week periods.

At the 4.5% protein level the values ranged in the following descending order: Oats, rye, polished rice, brown rice. Corn, barley, hard and soft wheat showed about the same value, which was lower than that of the other cereals.

Brown rice surpassed all of the others at the 7.5% level. Rolled oats and rye gave much higher values than corn, wheat and barley.

At the 9.5% level oats outranked wheat and rye. Hard and soft wheat gave practically the same values.

A strikingly higher value was obtained for oats than for hard wheat at the 12% protein level—the only two that could be compared at this level.

Oats, rice and rye proved superior to all of the other cereals at the protein levels at which they could be compared.

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# THE NUTRITIVE VALUE OF CEREAL PROTEINS IN HUMAN SUBJECTS<sup>1</sup>

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Large quantities of cereals are used directly as human food in this country. For example, in 1944, 46 million bushels of oats and 12 million bushels of corn were consumed as breakfast cereals (U. S. D. A., '46, pp. 46, 56). In the 5-year period 1936-1941 the average annual per capita consumption of breakfast cereals was almost 10 pounds (9.6 lbs.) (U. S. D. A., '42, p. 657).

Several studies of the nutritive value of cereal proteins have been made with human subjects (Mendel and Fine, '11a, b, c; Murlin and Mattill, '38; Murlin, Nasset and Marsh, '38). The latter authors concluded that certain types of heat treatment, particularly the explosion or puffing process, decrease the nutritive value of cereal proteins, as measured by their ability to maintain nitrogen equilibrium. In these studies the cereals were compared with egg protein as the standard protein. In the present investigation certain oat, corn and wheat cereals were compared with the unprocessed cereal with respect to their ability to maintain nitrogen equilibrium in the adult human.

<sup>1</sup> These studies were aided by a grant from the Nutrition Department of General Mills, Inc.

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## EXPERIMENTAL

Nitrogen balances were measured on adult human subjects consuming diets the protein of which was furnished primarily by cereals. From the change in the balances, replacement values were calculated by the method of Murlin and Mattill ('38).

The materials used for this study were:

1. Rolled oats,<sup>3</sup> cooked as directed on the package and eaten immediately.

2. An exploded, ready-to-eat cereal,<sup>4</sup> composed of 70% oat flour and 20% corn and rye flours, eaten as it comes from the package. This product will hereafter be referred to as exploded oat cereal.

3. Unprocessed whole wheat. The wheat used was hard red spring wheat which was cracked at the mill and then ground at the laboratory. It was cooked with water and salt in the autoclave for 1 hour at 15 lb. steam pressure.

4. Wheat flakes,<sup>5</sup> eaten as they come from the package.

5. Exploded wheat.<sup>6</sup> Some difficulty was encountered in consuming the large volume of this product until the subjects found it possible to "de-explode" the cereal with water and slight pressure.

6. Yellow hybrid field corn. The entire lot of corn was washed, dried for several days in a stream of warm air and thoroughly mixed. It was stored in air-tight containers in the refrigerator until ready for use. Before eating, the corn was either ground and cooked with water and salt, or cooked with water and salt without grinding. The cooking was done in the autoclave for 1 hour at 15 lb. steam pressure.

7. An oven-expanded, ready-to-eat corn cereal.<sup>7</sup> This product was eaten as it came from the package.

<sup>3</sup> Quaker Oats, manufactured by the Quaker Oats Company.

<sup>4</sup> Cheerioats, produced by General Mills, Inc. This product is now known as Cheerios.

<sup>5</sup> Wheaties, made by General Mills, Inc.

<sup>6</sup> Quaker Puffed Wheat Sparkies, made by Quaker Oats Company.

<sup>7</sup> Kix, made by General Mills, Inc.

8. Corn flakes,<sup>8</sup> eaten as it came from the package.

All of these products, with the exception of the whole wheat and the corn, were purchased from wholesale grocers in Cleveland. The wheat was purchased from the Montana Flour Mills in Cleveland, and the corn from Kohl and Sons Feed Store, Parma Heights, Ohio. Analyses of the cereals used are shown in table 1.

Vitamins were supplied by multiple vitamin capsules,<sup>9</sup> 1 capsule daily furnishing: vitamin A 5000 U.S.P. units, vitamin

TABLE 1  
*Analyses of products used.*

PRODUCT	MOISTURE	NITROGEN
	%	%
Milk-cream mixture	....	0.47
Rolled oats	7.90	2.59
Exploded oat cereal	5.62	2.17
Whole wheat	13.38	2.42
Wheat flakes		
Lot 1915	4.53	1.65
Lot 2185	5.83	1.60
Exploded wheat	5.26	2.24
Field corn	12.73	1.36
Expanded corn cereal		
Lot 2845	5.04	1.25
Lot 2955	4.97	1.25
Lot 2965	5.12	1.26
Corn flakes	2.89	1.22

D 500 U.S.P. units, thiamine hydrochloride 1.5 mg, riboflavin 2 mg, nicotinamide 20 mg, pyridoxine hydrochloride 0.5 mg, calcium pantothenate 5 mg, and ascorbic acid 37.5 mg. On the corn diets, the vitamin capsules<sup>10</sup> supplied daily: vitamin A 10,000 U.S.P. units, vitamin D 1000 U.S.P. units, thiamine hydrochloride 5 mg, riboflavin 5 mg, nicotinamide 25 mg, pyridoxine hydrochloride 1.5 mg, calcium pantothenate 5 mg,

<sup>8</sup> Kellogg Corn Flakes, made by the Kellogg Company.

<sup>9</sup> Abbott's Vitakaps Improved, made by Abbott Laboratories.

<sup>10</sup> Abbott's Dayamins, made by Abbott Laboratories.

and ascorbic acid 100 mg. Salt and water were allowed ad libitum.

On the first 2 experiments with rolled oats and exploded oat cereal, additional protein was supplied as a 1:1 mixture of milk and cream (20%). Calories needed in addition to the cereal and milk were supplied by butter (or oleomargarine) or sucrose or both.

The diets were designed to provide 0.70 gm of protein ( $N \times 6.25$ ) and approximately 37.5 Cal. per kg of body weight per day. In the first experiment on rolled oats and exploded oat cereal, the cereal supplied approximately 55% of the total nitrogen intake. In the second experiment on the same cereals, the cereal nitrogen supplied 80% of the total dietary nitrogen. In the remainder of the experiments, the cereal supplied, as nearly as possible, all of the dietary nitrogen. The diets are summarized in table 2.

A total of 8 subjects (3 women, 5 men) were used in these studies. They were all white adults, all except one having had training in biochemistry, and all aware of the importance of accurate and complete urine and stool collections and careful weighing of the daily diets. They cooperated well in consuming the prescribed diets, in abstaining completely from food and drinks not prescribed, and in reporting the almost inevitable occasional loss of part of a urine specimen. Throughout the experiments the subjects continued in their normal activities.

The diets were eaten for a period of 2 days before collections were begun. On the morning that collections were started, a stool marker (charcoal or barium sulfate) was ingested before breakfast, and urine collections begun. Stool collections were started when the marker appeared, including the marked stool with the collection. At the end of the experimental period, another marker was ingested on the morning that the last urine sample was completed and stool collections continued until the marker appeared, not including the marked stool with the collections. The subjects were

TABLE 2  
Summary of daily diets.

SUBJECT	CEREAL	MILK-CREAM MIXTURE	SUCROSE	BUTTER	NITROGEN
	gm	ml	gm	gm	gm
Exp. 1	Exploded oat cereal				
G.G.	153	628	206	0	6.26
L.G.	136	554	183	0	5.55
R.L.	160	646	215	0	6.51
C.K.	207	834	280	0	8.41
W.P.	157	634	212	0	6.38
M.P.	189	768	253	0	7.71
	Rolled oats				
G.G.	152	628	204	0	6.88
L.G.	135	554	181	0	6.10
R.L.	159	646	213	0	7.15
C.K.	206	834	276	0	9.25
W.P.	156	634	210	0	7.02
M.P.	187	768	251	0	8.45
Exp. 2	Exploded oat cereal				
G.G.	237	272	223	0	6.42
L.G.	212	246	200	0	5.75
R.L.	250	292	234	0	6.80
C.K.	317	366	295	0	8.59
W.P.	240	276	227	0	6.49
G.S.	291	336	274	0	7.89
	Rolled oats				
G.G.	199	272	250	0	6.42
L.G.	178	246	224	0	5.75
R.L.	210	292	262	0	6.80
C.K.	266	366	332	0	8.59
W.P.	201	276	254	0	6.49
G.S.	244	336	306	0	7.89
Exp. 3	Exploded oat cereal				
A.F.	436	0	327	0	9.46
C.K.	388	0	177	80	8.42
G.S.	360	0	38	142	7.81
	Rolled oats				
A.F.	366	0	375	0	9.46
C.K.	325	0	222	80	8.42
G.S.	302	0	76	142	7.81
Exp. 4	Whole wheat				
A.F.	404	0	390	0	9.80
C.K.	356	0	194	113	8.63
G.S.	361	0	184	120	8.75
	Wheat flakes 1915				
A.F.	594	0	197	0	9.80
C.K.	523	0	0	125	8.63
G.S.	530	0	0	125	8.75
	Wheat flakes 2185				
A.F.	612	0	180	0	9.80
C.K.	540	0	0	117	8.63
G.S.	547	0	0	117	8.75
	Exploded wheat				
A.F.	437	0	345	0	9.80
C.K.	385	0	151	114	8.63
G.S.	391	0	96	150	8.75
Exp. 5	Field corn				
A.F.	721	0	93	0	9.80
C.K.	635	0	0	76	8.63
G.S.	644	0	142	0	8.75
	Expanded corn cereal				
A.F.	784	0	45	0	9.80
C.K.	690	0	0	55	8.63
G.S.	700	0	0	53	8.75
	Corn flakes				
A.F.	804	0	27	0	9.80
C.K.	707	0	0	46	8.63
G.S.	717	0	84	0	8.75

weighed daily after completing a 24-hour urine sample and before breakfast.

The urines were collected directly into bottles containing toluene as a preservative. The 24-hour samples were mixed, volume and specific gravity measured, and an aliquot removed for analysis. The stool collections were made directly into wide mouth mason jars having lids sealed with rubber rings. All samples were kept under refrigeration until analyzed.

The urine samples were analyzed for total nitrogen and creatinine. Total nitrogen determinations were run by a semi-micro Kjeldahl distillation-titration procedure, using copper sulfate or selenium, or both, as catalyst. Urine creatinine was measured by an adaptation of the Folin ('14) colorimetric procedure to the Evelyn photoelectric colorimeter. In those urine samples, reported as being incomplete, in which the creatinine content showed a marked deviation from the mean daily creatinine excretion of that subject, a correction was applied. The actual nitrogen figure obtained by analysis was multiplied by the ratio mean daily creatinine/creatinine in sample to estimate the total nitrogen in the complete sample. In a total of 462 daily urine collections, there were 4 incomplete samples, of which 3 occurred in the first experiment.

The stool specimens for each subject were pooled, partially digested with sulfuric acid on the steam bath, cooled, made up to volume and sampled with mixing (Peters and Van Slyke, '32). Total nitrogen was estimated as with the urine samples.

#### RESULTS AND DISCUSSION

In the first experiment 3 subjects (L.G., M.P., W.P.) started on the exploded oat cereal diet (table 2) and 3 subjects (G.G., R.L., C.K.) started on the rolled oats diet (table 2). These diets were continued until collections had been made for 9 days, at which time a stool marker was ingested and the 2 groups changed diets, those who had been eating the exploded oat cereal substituting rolled oats and *vice versa*. Urine and stool collections were continued for 9 more days. The mean daily nitrogen balances observed in these subjects are pre-

sented in the upper part of table 3. It will be seen that in every case the nitrogen balance is more favorable on the rolled oats diet than it is on the exploded oat cereal diet. From this, it might be inferred that the availability of the nitrogen of the exploded oat cereal was less than that of rolled oats. However,

TABLE 3

*Mean daily nitrogen balances (all figures are grams of nitrogen per day).*

SUBJECT <sup>1</sup>	EXPLODED OAT CEREAL				ROLLED OATS			
	Intake	Urine	Stool	Balance	Intake	Urine	Stool	Balance
A. Experiment 1. Cereal furnished 55% of dietary nitrogen.								
G.G.	6.26	5.15	1.49	-0.38 ± .48 <sup>2</sup>	6.88	5.87	1.32	-0.31 ± .33 <sup>2</sup>
R.L.	6.51	5.50	0.87	+0.14 ± .54	7.15	5.23	0.82	+1.10 ± .69
C.K.	8.41	8.52	1.31	-1.42 ± .85	9.25	9.13	1.24	-1.12 ± .42
L.G.	5.55	6.36	0.84	-1.64 ± .29	6.10	5.66	1.06	-0.62 ± .58
M.P.	7.71	7.24	1.47	-1.00 ± .83	8.45	6.92	1.52	+0.01 ± .58
W.P.	6.38	7.25	1.42	-2.29 ± .69	7.02	6.55	1.59	-1.12 ± .38
Mean	6.80			-1.10	7.47			-0.34
B. Experiment 2. Cereal furnished 80% of dietary nitrogen.								
G.G.	6.42	5.12	1.51	-0.21 ± .29	6.42	5.97	1.40	-0.95 ± .16
R.L.	6.80	5.03	1.87	-0.10 ± .31	6.80	5.21	0.73	+0.86 ± .31
C.K.	8.59	7.05	1.93	-0.39 ± .27	8.59	7.36	1.47	-0.24 ± .44
L.G.	5.75	5.65	0.68	-0.58 ± .27	5.75	6.53	1.45	-2.23 ± .31
G.S.	7.89	8.23	1.81 <sup>3</sup>	-2.15 ± .57	7.89	6.40	1.89	-0.40 ± .29
W.P.	6.49	7.42	1.50 <sup>3</sup>	-2.43 ± .55	6.49	6.61	1.50	-1.62 ± .37
Mean	6.99			-0.98	6.99			-0.76

<sup>1</sup> The sex, age, height (cm), weight (kg), respectively, of the subjects are as follows: G.G., F, 29, 161, 57.2; L.G., F, 29, 167, 51.5; R.L., F, 21, 163, 60.7; C.K., M, 29, 181, 77.5; W.P., M, 37, 173, 58.2; M.P., M, 29, 170, 72.9; G.S., M, 26, 186, 74.8; A.F., M, 31, 175, 85.6.

<sup>2</sup> Standard deviation of mean.

<sup>3</sup> Estimated from digestibility as shown in table 4.

in every case the nitrogen intake was also higher on the rolled oats diet than it was on the exploded oat cereal diet, and all of the increased intake represented rolled oats nitrogen. The more favorable balance (mean 0.76 gm nitrogen per day) on the rolled oats diet was undoubtedly due, at least in part, to the increased nitrogen intake (mean 0.67 gm nitrogen per



day) on the rolled oats diet. The data thus obtained do not permit any definite conclusion with regard to the relative utilization of the nitrogen of these 2 cereals.

On the basis of the data reported by Stewart et al. ('43) on growing rats, it was expected that a large difference in the utilization of the nitrogen of these 2 products would be apparent. Since inconclusive results were obtained on the first experiment, it was decided to repeat the experiment with more

TABLE 4

*Experiment 2.*

*Estimation of fecal nitrogen for G.S. and W.P. on exploded oat cereal diet.*

SUBJECT	DRY MATTER INTAKE <sup>1</sup>	METABOLIC NITROGEN <sup>2</sup>	FECAL NITROGEN	DIGESTIBILITY <sup>3</sup>	ESTIMATED FECAL NITROGEN
	<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>%</i>	<i>gm</i>
G.G.	504	1.16	1.51	94.5	...
R.L.	531	1.22	1.87	90.5	...
C.K.	671	1.54	1.93	95.5	...
L.G.	452	1.04	0.68	100	...
G.S.	619	1.42	...	...	1.81
W.P.	511	1.18	...	...	1.50
			Mean	95.1	

<sup>1</sup> Dry matter calculated using the data of tables 1 and 2 and assuming 21% solids in the milk-cream mixture.

<sup>2</sup> Fecal metabolic nitrogen calculated assuming 0.23 gm per 100 gm dry matter consumed (Mitchell and Hamilton, '29, p. 234).

<sup>3</sup> Per cent digestibility =  $\frac{[\text{Food N} - (\text{Fecal N} - \text{Metabolic N})]}{\text{Food N}} \times 100$ . (Hawk et al., '47, p. 961.)

of the protein of the diet being supplied by the cereal and less from the milk-cream mixture.

The plan of the second experiment was the same as that of the first, except that the cereal supplied 80% of the dietary nitrogen and the experimental period was shortened to 4 days' collection on each diet. Three of the subjects (L.G., W.P., G.S.) started on the exploded oat cereal diet, and 3 (G.G., R.L., C.K.) started on the rolled oats diet.

The mean daily nitrogen balances observed are presented in the lower part of table 3. In the case of subjects G.S. and

W.P., the stool specimens on the exploded oat cereal diet were lost before they had been analyzed, and consequently the fecal nitrogen for these 2 subjects had to be estimated indirectly. This was done by making use of the digestibility of the exploded oat cereal-milk protein mixture calculated from the intakes of the other 4 subjects as shown in table 4. It will be seen that in 4 of the 6 subjects, the nitrogen balance on rolled oats and milk was more favorable than the nitrogen balance on exploded oat cereal plus milk, but the mean replacement value (table 5) shows a very slight difference between the 2

TABLE 5  
*Experiment 2.*  
*Calculation of replacement value.*

SUBJECT	NITROGEN BALANCE ON		DIFFERENCE IN BALANCE	NITROGEN INTAKE	REPLACE- MENT VALUE
	Rolled oats + milk	Exploded oats + milk			
	(1)	(2)		(4)	(5) <sup>2</sup>
G.G.	- 0.95	- 0.21	+ 0.74	6.42	112
R.L.	+ 0.86	- 0.10	- 0.96	6.80	86
C.K.	- 0.24	- 0.39	- 0.15	8.59	98
L.G.	- 2.23	- 0.58	+ 1.65	5.75	129
G.S.	- 0.40	- 2.15	- 1.75	7.89	78
W.P.	- 1.62	- 2.43	- 0.81	6.49	88
				Mean	98.5

<sup>1</sup> Column (2) minus column (1).

<sup>2</sup> Column (3) expressed as a per cent of column (4). If column (3) is positive, the per cent is added to 100; if negative, subtracted from 100.

protein mixtures. It is thus doubtful if there is any real difference in availability of the protein of exploded oat cereal plus milk as compared with that of rolled oats plus milk.

There still being some doubt in our minds with regard to the significance of the results, it was decided to try another experiment in which the supplementing effect, if any, of the milk protein was absent. Three white adult male subjects (A.F., C.K., G.S.) were put on diets (table 2) in which all of the protein was furnished by the cereal. The exploded oat cereal diets were eaten for a total of 12 days, on the last 10 of

which urine and stool collections were made.<sup>11</sup> At the end of the 12-day period, a stool marker was ingested and a daily supplement of 5 gm DL-lysine monohydrochloride included in the diet for an additional 5-day period. The lysine was mixed with the food after cooking so that the supplement would be taken at the same time as the food and not in a single dose. Berg and Rose ('29) have observed that amino acid supplements are more effective if taken in divided doses, rather than in a single dose. After a rest period of 11 days, exactly the same procedure was applied to rolled oats. Lysine was chosen as the amino acid supplement because it is the first limiting essential amino acid in these cereals.<sup>12</sup>

The results are summarized in the first part of table 6. In every case, the addition of lysine to the exploded oat cereal diet improved the nitrogen balance, the mean increase being 1 gm per day. This would appear to indicate that the amount of the exploded oat cereal consumed furnished insufficient lysine for the maintenance of nitrogen equilibrium in adult human subjects. In the same way, addition of lysine to the rolled oats diet increased the nitrogen balance an average of 0.96 gm per day. The rolled oats, in the quantities consumed, would therefore also appear to furnish insufficient lysine for nitrogen equilibrium in adult humans.

The replacement values are calculated as shown in table 7. It would seem that the replacement value of exploded oat cereal nitrogen, in terms of rolled oats nitrogen (101%), indicates no significant difference in the utilization of the nitrogen of these 2 cereals by the adult human when they are consumed in quantities to furnish 0.112 gm nitrogen per kg of body weight per day. Similarly, the replacement value of exploded oat cereal plus lysine, in terms of rolled oats plus lysine (101%), when ingested in these quantities, would ap-

<sup>11</sup> In the case of subject G.S., the figures used represent only the last 7 days of collection, since on the third day of collection he had a gastrointestinal upset and was unable to eat his full quota of food. On the morning of the fourth day, he ingested another marker to separate the stool of the first 3 days from that of the remainder of the experiment.

<sup>12</sup> Personal communication from Dr. Lela E. Booher, General Mills, Inc.

TABLE 6  
*Summary of mean daily nitrogen balances (all figures are grams of nitrogen per day).*

DIET	A.F. <sup>1</sup>			O.K.			G.S.			MEAN BALANCE
	Urine	Stool	Balance	Urine	Stool	Balance	Urine	Stool	Balance	
Exploded oat cereal	9.24	2.12	-1.90 ± .92 <sup>1</sup>	7.13	1.49	-0.20 ± .35 <sup>1</sup>	7.33	1.88	-1.40 ± .99 <sup>1</sup>	-1.17
Exploded cereal + lysine	8.40	2.32	-0.50 ± .87	7.06	1.71	+0.41 ± .23	6.96	2.03	-0.42 ± .33	-0.17
Rolled oats	8.93	2.59	-2.06 ± 1.06	7.13	1.67	-0.38 ± .52	6.69	2.33	-1.21 ± .32	-1.22
Rolled oats + lysine	8.43	2.46	-0.67 ± .80	7.45	1.65	+0.08 ± .43	6.78	1.97	-0.18 ± .91	-0.26
Whole wheat	8.63	2.67	-1.50 ± .71	7.02	1.95	-0.34 ± .35	7.03	2.02	-0.30 ± .50	-0.71
Wheat flakes	9.15	2.51	-1.74 ± .58	7.32	2.25	-0.94 ± .45	7.36	2.29	-0.90 ± .32	-1.19
Exploded wheat	11.24	2.49	-3.92 ± .73	7.52	2.28	-1.17 ± .55	7.75	2.56	-1.56 ± .50	-2.22
Field corn	7.87	3.64	-1.71 ± .69	6.64	3.59	-1.60 ± .57	7.71	2.52	-1.48 ± .65	-1.60
Expanded corn cereal	8.99	3.31	-2.50 ± .65	6.99	2.57	-0.93 ± .33	7.05	2.93	-1.22 ± .55	-1.55
Corn flakes	8.78	3.39	-1.74 ± .74	7.15	2.77	-1.28 ± .48	7.24	2.89	-1.38 ± .53	-1.67

<sup>1</sup> Standard deviation of mean.

TABLE 7  
*Calculation of replacement values.*

SUBJECT	BALANCE ON		DIFFERENCE <sup>1</sup>	NITROGEN INTAKE	REPLACE- MENT VALUE <sup>2</sup>
A Oat cereals					
	Rolled oats	Exploded oats			
A.F.	— 2.06	— 1.90	+ 0.16	9.46	102
C.K.	— 0.38	— 0.20	+ 0.18	8.42	102
G.S.	— 1.21	— 1.40	— 0.19	7.81	98
				Mean	101
	Rolled oats + lysine	Exploded oats + lysine			
A.F.	— 0.67	— 0.50	+ 0.17	10.22	102
C.K.	+ 0.08	+ 0.41	+ 0.33	9.18	104
G.S.	— 0.18	— 0.42	— 0.24	8.57	97
				Mean	101
B Wheat cereals					
	Whole wheat	Wheat flakes			
A.F.	— 1.50	— 1.74	— 0.24	9.80	98
C.K.	— 0.34	— 0.94	— 0.60	8.63	93
G.S.	— 0.30	— 0.90	— 0.60	8.75	93
				Mean	95
	Whole wheat	Exploded wheat			
A.F.	— 1.50	— 3.92	— 2.42	9.80	75
C.K.	— 0.34	— 1.17	— 0.83	8.63	90
G.S.	— 0.30	— 1.56	— 1.26	8.75	86
				Mean	84
C Corn cereals					
	Field corn	Expanded corn			
A.F.	— 1.71	— 2.50	— 0.79	9.80	92
C.K.	— 1.60	— 0.93	+ 0.67	8.63	108
G.S.	— 1.48	— 1.23	+ 0.25	8.75	103
				Mean	101
	Field corn	Corn flakes			
A.F.	— 1.71	— 2.36	— 0.65	9.80	93
C.K.	— 1.60	— 1.28	+ 0.32	8.63	104
G.S.	— 1.48	— 1.38	+ 0.10	8.75	101
				Mean	99

<sup>1 2</sup> See footnotes to table 5.

pear to indicate no significant difference in the utilization of these nitrogen sources by adult human subjects.

Supplementation of the 2 oat cereal diets with lysine brings about an improvement in the nitrogen balance in each case, amounting to from 0.46 to 1.40 gm of nitrogen per day. Five grams of DL-lysine monohydrochloride contains 0.76 gm of nitrogen. If it is assumed that in the human, as in the rat, only the L-isomer is physiologically available (Berg, '36), then the addition of 0.38 gm of nitrogen as L-lysine brought about the retention of from 0.08 to 1.02 gm of "extra" nitrogen (or from 21 to 268%) in addition to that of the L-lysine.

The mean improvement in the nitrogen balance on the addition of lysine was very nearly the same whether the lysine was added to the exploded oat cereal diet or the rolled oats diet. This would seem to indicate that the degree of lysine deficiency was approximately the same on the exploded oat cereal diet as on the rolled oats diet. Amino acid analyses (Mitchell and Block, '46, table 9) show the 2 cereals to have about the same lysine content.

It would thus appear that the processing of the exploded oat cereal had no significant effect on the nutritive value of the nitrogen of this cereal so far as adult human subjects are concerned.

The similarity in nutritive value of the proteins of these 2 oat cereals appears to be in disagreement with the findings reported by Stewart et al. ('43). These workers observed, in growing white rats, only 32 to 39% as good gain in weight per gram of protein eaten with the exploded oat cereal as with rolled oats. There are 2 important differences between the present study and that of Stewart et al. In the present study *adult human* subjects were used, whereas Stewart et al. used *growing albino rats*.

It is quite generally conceded that the nutritive value of a protein or mixture of proteins depends upon the mixture of amino acids made available to the organism as a result of the ingestion of that protein (see e.g., Jackson, '45, p. 987). It is conceivable that there is a quantitative difference in the

amino acid requirements for growth and for maintenance (Jackson, '45, p. 991; Neuberger and Webster, '45), and also there may well be a qualitative difference (Almquist, '45, p. 1271). In light of this observation along with a consideration of species differences in amino acid requirements (see e.g., Womack and Kade, '44, pp. 223-229), the results of the 2 studies may not be in radical disagreement. The depressed nutritive value, for the growing rat, of the protein of exploded oat cereal as compared with rolled oats (Stewart et al., '43; Mitchell and Block, '46) apparently is due to some factor other than the amino acid content. Mitchell and Block ('46) suggest several possible explanations. In any case, the ultimate criterion of nutritive value for humans must be the results of studies done on human subjects.

In the fourth experiment the same 3 subjects were used for a comparison of the nitrogen balances on 2 prepared wheat cereals and unprocessed whole wheat. The diets (table 2) were eaten for a total of 14 days each, with complete urine and stool collections for the last 12 days.<sup>13</sup> The exploded wheat diet was eaten first, followed, after a rest period of a week, by the wheat flakes diet. After another rest period of 11 days, the whole wheat diet was eaten.

The observed nitrogen balances are summarized in table 6. It will be seen that, with all of the 3 subjects, the most favorable balance was observed on the whole wheat diet, there was an intermediate value on the wheat flakes diet, and the most unfavorable balance was observed on the exploded wheat diet. The difference between the mean balance on wheat flakes and the mean balance on whole wheat (0.48) was only a third as great as the difference between the mean balance on the exploded wheat and the mean balance on the whole wheat (1.51). The replacement values (table 7, section B) also show this difference between the 2 processed wheat cereals. It would

<sup>13</sup> In the case of subject A.F. on the wheat flakes diet, the figures for only 11 days were used, since on one of the days he had gastrointestinal upset with diarrhea and vomiting, and did not eat his full quota of food. The following morning he felt equal to continuing the experiment and ingested an extra fecal marker to separate the stool for the 2 periods.

appear that the processing brought about a greater decrease in the nutritive value of the nitrogen in the exploded wheat than in the wheat flakes. These findings are in agreement with the results reported by Murlin, Nasset and Marsh ('38) who found the protein of exploded wheat to be definitely inferior to that of wheat flakes.

The final experiment was a comparison of the nitrogen balances of the same 3 subjects on 2 processed corn cereals and unprocessed whole corn. The first diet to be eaten was the expanded corn cereal diet, which was followed by the corn flakes diet after a rest period of about a month. This was

TABLE 8

*Summary of nitrogen balances for 3 subjects (all figures are grams of nitrogen per day).*

CEREAL	MEAN N INTAKE	MEAN N BALANCE
Whole wheat	9.06	— 0.71
Exploded oat cereal <sup>1</sup>	8.56	— 1.17
Wheat flakes	9.06	— 1.19
Rolled oats <sup>1</sup>	8.56	— 1.22
Expanded corn cereal	9.06	— 1.55
Whole corn	9.06	— 1.60
Corn flakes	9.06	— 1.67
Exploded wheat	9.06	— 2.22

<sup>1</sup> The position of the oat cereals in the table is probably influenced by the nitrogen intake. See text for discussion.

followed, after a rest period of from 23 to 52 days, by the whole corn diet.

The mean daily nitrogen balances observed are shown in table 6. The mean balance for the 3 subjects was best on the expanded corn cereal diet, intermediate on the field corn diet and poorest on the corn flakes diet. The replacement values (table 7, section C) indicate little, if any, significant difference in the nitrogen of these 3 cereals in their ability to maintain nitrogen equilibrium in adult human subjects.

An estimate of the relative value of the proteins of the unprocessed cereals can be obtained since, on the last 3 experi-



ments, the same 3 subjects were used. The mean balances are presented in table 8 in decreasing order. Of the unprocessed cereals, it will be seen that in these experiments the best balance was observed on whole wheat, followed by rolled oats and then corn. It is generally accepted that the protein of oats has a higher nutritive value than that of wheat (see, e.g., Wilder and Keys, '42). The variance of the present results may be due to the lower nitrogen intake on the oat cereals, since the difference in nitrogen intake (0.50 gm per day) was equal to the maximum difference in nitrogen balance (0.51 gm per day).

#### CONCLUSIONS

The following conclusions appear justified on the basis of the data presented.

1. Rolled oats and exploded oat cereal, as sources of nitrogen, are equally well utilized by adult human subjects when they furnish 55, 80 or 100% of the total dietary nitrogen, the remainder of the nitrogen being furnished by milk, when the total protein ( $N \times 6.25$ ) intake is approximately 0.70 gm per kg body weight per day, and the Caloric intake approximately 37.5 Cal. per kg body weight per day.

2. Both of these cereals, at this level of intake, furnish suboptimal amounts of lysine, and the degree of lysine deficiency of the 2 cereals is the same within the limits of error of the method used.

3. Both wheat flakes and exploded wheat are inferior to unprocessed whole wheat in the maintenance of nitrogen equilibrium. The wheat flakes, however, are definitely better in this respect than the exploded wheat.

4. The nitrogen of the expanded corn cereal and corn flakes is practically identical with that of unprocessed whole corn in its ability to maintain nitrogen equilibrium in the adult human.

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# THE ROLE OF THYROID ACTIVITY IN THE PATHOGENESIS OF HEPATIC LESIONS DUE TO CHOLINE AND CYSTINE DEFICIENCY

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TWO FIGURES

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## INTRODUCTION

The possibility that increased activity of the thyroid gland might have a deleterious effect on the liver in man was first suggested by Paul in 1865. During the intervening years there have been numerous allusions to a thyroid-liver relationship, the exact nature of which has never been very clearly defined, nor have morphological changes in the liver during life (Piper and Paulsen, '47) or at autopsy (Weller, '33) been at all constant. In experimental animals, the administration of thyroid substance or thyroxine at high dosage levels for a relatively short period has not resulted in liver changes other than those usually seen in starvation; moderate enlargement has been observed in longer experiments where lesser amounts of thyroid were administered (McIver, '42).

Choline deficiency in the rat leads to extreme fatty infiltration of the liver, followed by focal areas of necrosis and fibrosis; on the other hand, cystine deficiency results in acute, massive, hepatic necrosis without fatty infiltration (György, '44; Glynn et al., '45; Handler and Dubin, '46). It seemed of

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interest, therefore, to determine whether the effects of choline or cystine deficiency could be modified by varying the level of thyroid activity. That this may occur has already been indicated by the observations of György and Goldblatt ('45) that thiouracil, which interferes with the formation of the active principle of the thyroid, inhibits the development of cirrhosis in choline-deficient rats.

#### EXPERIMENTAL

The animals used in these experiments were male rats of the Vanderbilt strain (Wolfe, Bryan and Wright, '38). They were placed on the experimental regimes when they had attained a weight of 50 gm, and housed in individual cages. All experiments were terminated after 120 days unless otherwise stated. At this time the animals were sacrificed by carotid section and livers and thyroids removed and preserved in formalin. Blocks were embedded in paraffin; sections were cut and then stained with hematoxylin and eosin.

In estimating the degree of change, i.e., fatty infiltration, scarring and necrosis, all of the sections have been graded arbitrarily 1 to 4 plus on the basis of the degree of alteration present. The final estimation is a summary of the total number of animals in each group. In view of the rather wide variations in morphological changes from animal to animal, such a mode of expression is certainly not ideal. It would seem, however, the only suitable way of summarizing the data and is similar to the procedure used earlier (Handler and Dubin, '46). It should be pointed out that the variations in the response of the liver to choline-deficient diets are notorious. In our experience, based on studies of various mineral and vitamin deficiencies in rats, choline deficiency is unique in the inconstancy of the final morphological picture.

#### *Thyroid activity and hepatic damage in choline-deficient rats*

The basal ration used for this study was patterned after that previously found in this laboratory to be most effective

in producing choline deficiency in rats (Handler and Dubin, '46). It had the following percentage composition: casein 5, starch 60, cotton oil 10, lard 15, cod liver oil 5, cystine 0.5, cholesterol 0.5 and salts (Hubbell, Mendel and Wakeman, '37) 4. In addition, the diet contained in milligrams per kilo, the following vitamins: thiamine 3, riboflavin 5, pyridoxine 3, calcium pantothenate 30, niacin 100, naphthohydroquinone acetate 5, biotin 1, folic acid 2,  $\alpha$ -tocopherol 50. The additional supplements to this ration and the results are summarized in table 1.

The history of the animals in group A was similar to that of previous rats on such diets (Handler and Dubin, '46). At autopsy the livers of these animals contained large amounts of fat. In addition, there were areas of varying size where the liver cells had become necrotic and had been replaced by connective tissue. In many of the livers, large areas of scarring were encountered so that the picture was not entirely that of nodular cirrhosis as had been encountered before when this diet was employed. Examination of the thyroid glands of this group revealed hyperplasia in 3 of the animals examined.

Hypothyroidism induced by thyroidectomy and by thiouracil or p-aminobenzoic acid feeding appeared to protect rats against the deleterious effects of choline deficiency. The results obtained with the thyroidectomized rats in group B were not very convincing since almost all the animals died before the termination of the experiment, albeit somewhat later than the control rats of group A. Unfortunately, the livers of these animals were so badly autolyzed that satisfactory histological examination was possible in only 2 of this group. Death may have been entirely unrelated to the choline deficiency since a control, choline-fed group, not shown in the table, also died after about 100 days. It does seem significant that no fat, necrosis, or scarring was observed in the 2 livers of group B rats which were suitable for examination. Of the other 2 agents, thiouracil which produced the more extensive hyperplasia of the thyroid was also most effective in protecting

hepatic necrosis and fibrosis and, perhaps most significant, only 1 of 8 rats died during the experimental period.

The results of thyroid feeding in this, as in the subsequent experiments, were rather inconclusive. All of the animals died, and much sooner than did those on the control diet. However, there was great variation in the amount of fat and necrosis observed in their livers. The rats that died soonest showed very little fat or necrosis, while the livers of the animals living longest contained more fat and were somewhat more necrotic; however, none approached the lipid content of the controls. Only 1 liver in this series was at all scarred, perhaps because the duration of life on this regime was too brief to permit any appreciable proliferation of fibrous tissue. The thyroid glands in this group, as was to be expected, were large and of the colloid type. Not included in table 1 were several groups of rats fed the basal ration to which considerably more thyroid powder had been added, in amounts up to 0.8% of the diet. These animals all died much more rapidly (7 to 20 days) and their livers showed no abnormalities.

Included also in table 1 are the results obtained when the fat content of the basal diet was lowered. Basal diet 2 contained 5% each of the cotton oil, lard and cod liver oil, a total of 15%, in contrast with basal diet 1 which contained 30% lipids. The relatively large amount of cod liver oil in these diets was deliberately included to enhance the deposition of ceroid (Wachstein, '45). Under these conditions, animals on the unsupplemented basal diet, group AA, were not as severely affected as those on the higher fat intake. Only one-half of the animals died and, while all animals showed very fatty livers, only moderate necrosis resulted and fibrosis was seen in only 1 of the 10 livers examined. Thyroid feeding at 0.1% of the diet did not hasten the demise of the animals, nor was there a differentiation between the livers of these rats and those of the unsupplemented controls. Increasing the thyroid dosage to 0.3% again resulted in early death of the animals but these showed variable amounts of liver fat and virtually no fibrosis or necrosis. And again, p-aminobenzoic acid, in large

amounts, produced morphological thyroid hyperplasia with complete protection against the necrotic and fibrotic processes although the livers were extremely fatty.

*Thyroid activity and hepatic damage in  
cystine-deficient rats*

In table 2 are presented the effects of varying levels of thyroid activity on the picture of acute cystine deficiency. Group A received basal ration 2 (low fat) while group B received basal ration 1 (high fat). No cystine was added to either ration. Thus, these 2 groups were both choline- and cystine-deficient. While the animals of group A (with 2 exceptions) died about 2 weeks before those of group B, their livers contained considerably less fat and were only patchily necrotic. In contrast, the necrosis in group B was not focal or central but involved great masses of liver. All further studies were made with the basal ration 1, without cystine, but with the supplements indicated in the table.

The addition of choline did not significantly delay death due to cystine deficiency but the livers of this group (E) did not seem quite as severely affected as those of group B. Thus, when groups A and E are compared with group B, it appears that the hepatic necrosis of cystine deficiency is more pronounced when conditions are such that the liver is concomitantly fatty, all other factors being held as constant as possible. Thyroid feeding accelerated the death of cystine-deficient rats (F) and also that of animals deficient in both cystine and choline (C). Both of these groups presented the dramatic picture of acute massive necrosis found in control group B even though they died several weeks earlier. This is in contrast to the behavior of the rats of group C, table 1. The latter group was deficient in choline, but not cystine, and their deaths were also accelerated by thyroid feeding, but their livers did not show the necrosis or fibrosis of choline deficiency. Thus, if the histological picture of the livers may be taken as a criterion, thyroid feeding accelerates the physiological events of cystine deficiency but not those of choline



TABLE 3

*Composition of diets used in study of hyperthyroidism.*

MATERIALS <sup>1</sup>	DIET NUMBER											
	1	2	3	4	5	6	7	8	9	10	11	12
Chow <sup>2</sup>	100	99	.	..	..	..	..	..	..	..	..	..
Cascein	..	..	30	30	30	30	30	5	5	5	5	5
Starch	..	..	50	49	49	30	29	60	59	59	60	59
Cotton oil	..	..	10	10	10	10	10	10	10	10	10	10
Lard	..	..	..	..	.	20	20	15	15	15	15	15
Salts <sup>3</sup>	..	..	4	4	4	4	4	4	4	4	4	4
Cod liver oil	..	.	5	5	5	5	5	5	5	5	5	5
Cystine	..	..	.	..	..	.	.	0.5	0.5	0.5	..	..
Cholesterol	..	..	..	..	..	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Choline	..	0.8	..	0.8	..	..	0.8	..	0.8	..	..	0.8
Methionine	..	..	..	..	1	..	..	..	..	1	..	..

<sup>1</sup> In addition all diets, except 1 and 2, contained the same vitamin supplement used with basal ration 1 (see text), but in doubled quantity.

<sup>2</sup> Purina Checkers, Ralston Co., St. Louis, Mo.

<sup>3</sup> Hubbell et al. ('37).

TABLE 4

*Effect of diet on mortality due to hyperthyroidism.*

DIET	0.4% THYROID			0.8% THYROID		
	No. of rats		Mean survival time <sup>1</sup>	No. of rats		Mean survival time <sup>1</sup>
	Total	Dead		Total	Dead	
			days			days
1	8	0	..	8	2	28
2	8	0	.	8	3	12
3	8	0	.	8	6	19
4	16	15	17	8	8	12
5	12	6	27	8	7	17
6	12	1	11	12	3	23
7	12	6	16	12	9	19
8	12	12	20	12	11	14
9	12	5	25	12	8	23
10	12	3	18	12	8	17
11	8	8	11	8	8	9
12	12	6	17	12	11	17

<sup>1</sup> Dead rats only.

choline partially protected against this (group 9) as did methionine (group 10). On a low protein, high fat ration deficient in cystine as well as choline (group 11), rats were more susceptible to hyperthyroidism than on any other ration studied, while the addition of choline to the diet appeared to offer some protection against both levels of thyroid feeding (group 12).

From these data it may be said that rats eating a commercial chow were less susceptible to experimental hyperthyroidism under these conditions than on any synthetic ration employed. On the whole, they were more resistant when eating high protein rather than low protein rations. No explanation can be offered for the increased susceptibility to hyperthyroidism which resulted from the addition of choline or methionine to an already adequate synthetic ration. On low protein diets, both choline and cystine deficiency markedly increased susceptibility to thyrotoxicosis, while animals deficient in both choline and cystine succumbed more rapidly than any other group in this study.

*The influence of preëxisting choline deficiency upon  
experimental hyperthyroidism*

One further experiment, summarized in table 5, was performed whose results are pertinent to this discussion. All rats were grown to 50 gm, housed in individual cages, and fed the cirrhosis-inducing basal ration 1 (described above) for 2 weeks. A representative group was sacrificed at this time and found to have a mean liver fat content of 24.3%, wet weight, range 17.1–29.8%. The remaining animals were subdivided into 4 groups. Group A remained on the same basal diet; group B was given the same diet plus 0.3% thyroid powder; group C received basal ration plus 0.3% thyroid powder and 0.8% choline. Group D was given basal ration plus 1% choline for 1 week and then offered the diet of group C. The entire experiment was then continued for 40 days. The results are summarized in table 5.

The results of such an experiment could not have been clear-cut since animals began to die of choline deficiency after

40 days. Nevertheless, the data certainly suggest that a pre-existing fatty liver affords some protection against the toxicity of thyroid feeding. While at first this would appear to be a contradiction of the findings shown in table 4 wherein choline modified the acute effects of thyroid feeding in animals on a

TABLE 5

*The effect of a preëxisting fatty liver on mortality due to hyperthyroidism.*

GROUP	SUPPLEMENTS TO DIET	NUMBER OF RATS		MEAN SURVIVAL TIME <sup>1</sup>
		Total	Dead	
				<i>days</i>
A	None	10	2	36
B	0.3% Thyroid	10	3	32
C	0.3% Thyroid 0.8% Choline	10	9	16
D	After 7 days on basal + 1% choline transferred to diet of group C	10	10	20

<sup>1</sup> Dead rats only.

All animals were fed basal ration 1 for 2 weeks in advance. At this time a representative group was found to have a mean liver fat content of 24.3%. The animals were then divided into 4 groups and continued on basal ration 1 but with supplements as indicated above.

low protein, high fat regime, it should be realized that at such levels of thyroid feeding the control group in that experiment (group 8, table 4) never developed fatty livers despite the choline deficiency. The data in table 5 do not suggest that choline deficiency, *per se*, protects against thyroid toxicity, but rather that a *preëxisting* fatty liver is less susceptible to thyroid toxicity than an otherwise normal liver.

#### DISCUSSION

The data which have been presented leave little doubt but that the physiological sequelae of choline and/or cystine defi-

ency are, in a considerable measure, determined by the level of thyroid activity. Hyperthyroidism, induced by feeding desiccated thyroid powder, definitely shortened the lives of rats on every diet used in this study. Cystine-deficient, choline-fed rats lived almost twice as long on the control diet as animals fed the same ration supplemented with thyroid powder. In the livers of both groups was found the acute massive necrosis previously described in cystine deficiency. In contrast, while choline-deficient, cystine-fed rats developed a focal or diffuse hepatic necrosis and fibrosis, the addition of thyroid powder to such a diet accelerated the demise of the experimental subjects, but their livers showed no specific change.

Since thyroid feeding accelerated the metabolic and histologic events of cystine deficiency it is not surprising that hypothyroidism (thiouracil feeding) delayed death due to cystine deficiency, and protected the liver cells. On the other hand, while hyperthyroidism cannot be stated to have definitely accelerated the metabolic events of choline deficiency, the various procedures calculated to induce hypothyroidism (thyroidectomy, thiouracil or p-aminobenzoic acid feeding) definitely protected rats against the hepatic necrosis and fibrosis of choline deficiency, at least for the experimental period employed in these studies. It is of interest that, while these livers showed little or no necrosis or fibrosis, they were, histologically, exceedingly fatty. Moreover, this could not have been due to a delayed accumulation of fat since hypothyroidism, even in experiments of but 21 days' duration, actually enhances the deposition of liver fat in choline-deficient rats (Handler, '47, '48). This, then, represents another instance in which the existence of a chronically fatty liver did not result in hepatic necrosis and fibrosis. That this may occur under other circumstances (nicotinamide feeding on a relatively high casein diet) has already been reported (Handler and Dubin, '46). The hepatic necrosis of choline deficiency, as usually seen, is therefore dependent upon at least a normal level of thyroid activity as well as upon the chronic fatty liver.

The experiment in which we attempted to determine the dietary conditions most propitious for death due to hyperthyroidism requires comment. No explanation is at hand to account for the relative resistance of the rats eating chow as compared to those eating our best synthetic ration. Whether this is due to the presence of some factor or factors not included in our rations, or to a different quantitative rather than qualitative composition of the known dietary factors, cannot be stated and remains a problem of potential significance in the management of hyperthyroid patients. From the results of Ershoff ('47) it appears likely that the chow used in this study may have contained the same factor, present in liver, which increases the resistance of rats to the effects of thyroid feeding. The increased susceptibility to hyperthyroidism which resulted from the addition of choline or methionine to a high protein ration, in contrast to the protection seemingly afforded when these were added to a low protein ration, also remains a confusing problem. However, it can definitely be stated that rats on low protein rations succumb to hyperthyroidism more rapidly than animals eating a high protein ration. Further protection is afforded if the high protein ration is also high in fat content rather than in carbohydrate although cystine deficiency, like choline deficiency, is more severe on high fat than low fat diets.

In contrast to clinical hyperthyroidism, simple experimental hyperthyroidism has not been found to be associated with liver damage (McIver, '42). However, 3 situations have already been described in the literature which may be said to predispose the liver to hepatic damage when the animals are also rendered hyperthyroid. Hyperthyroid, anoxic rats do show liver damage (McIver and Winter, '43); preëxisting liver damage (chloroform poisoning) is intensified by hyperthyroidism (McIver and Winter, '40); and hyperthyroid rabbits infected with either *S. aureus* or Shope papilloma virus (Seasly, '41, '42) also develop a hepatic necrosis. To this list, then, should be added 1 further possibility, i.e., improper diet. While, even now, it would be difficult to define the precise

dietary conditions necessary, it is obvious that a low protein ration, relatively deficient in cystine and choline, or their common precursor, methionine, may well be involved as well as a diet poor in the protective material found in commercial mixed rations and in liver. However, from the data presented herein, and from the fact that a relatively high protein diet protects the liver from the usual deleterious effects of fatty infiltration, it seems likely that hepatic damage due to hyperthyroidism is consequent upon an accelerated protein rather than carbohydrate metabolism. It remains possible, of course, that the acceleration of protein metabolism is a secondary effect of the hyperthyroidism and may be explained entirely by an accelerated demand for glyconeogenesis because of the increased rate of caloric expenditure. In summary, while simply hyperthyroidism may kill experimental animals without inducing frank hepatic damage, dietary imbalance is one of a number of predisposing influences which, when accompanied by hyperthyroidism, will lead to liver damage and death.

We have no explanation for the ameliorative action of sulfasuxidine, taurine and inositol against the hepatic necrosis and fibrosis of choline deficiency. None of these produced morphological thyroid hyperplasia, so their behavior should be differentiated from that of thiouracil and p-aminobenzoic acid. The sulfasuxidine may have acted in an indirect fashion by modifying the intestinal flora. Inositol did not operate *via* its lipotropic activity, as the livers of this group were as fatty as those of the others. It may be of some significance, however, that normal thyroid tissue contains a large concentration of inositol (Meyer, '46). The action of taurine remains entirely unexplained. Admittedly, however, these findings jeopardize our interpretation of the experiments employing thiouracil and p-aminobenzoic acid. While the latter agents definitely exerted an anti-thyroid action and the animals fed these compounds may justifiably be considered to have been in a relatively hypothyroid state, it may not be valid to attribute their beneficial effects to this aspect of their behavior. Until a rational explanation of the results obtained with

taurine, sulfasuxidine and inositol is available, it will remain possible that the results of thiouracil and p-aminobenzoic acid feeding, described herein, are non-specific. The chief justification for considering the results obtained in the present study as being due to the anti-thyroid action of these 2 compounds lies in the similar behavior of thyroidectomized rats.

The observation of thyroid hyperplasia in some of the choline-deficient as well as in certain of the choline- and cystine-deficient animals also requires some comment. Since this was not consistent, and did not occur at all in many of the animals, it would be hazardous to place much emphasis on it. It may be this variation in thyroid activity which underlies the inconsistency of the other morphological findings in choline deficiency. Further, it is not possible to state whether this thyroid hyperplasia is a direct result of a choline deficiency or whether it is secondary to events in the choline-deficient liver. In view of the readiness with which the thyroid responds to so many stimuli by morphologic hyperplasia, it would seem wise to reserve further comment until more pointed and extensive observations have been made.

#### SUMMARY

1. A decreased level of thyroid activity, induced by thyroidectomy, thiouracil or p-aminobenzoic acid feeding, prevents or retards the development of hepatic necrosis or fibrosis associated with choline and cystine deficiencies.

2. Although thyroid feeding hastened the death of choline-deficient rats, their livers contained little fat and showed no necrosis or fibrosis.

3. Thyroid feeding hastened the death of cystine-deficient rats; their livers exhibited the acute necrosis characteristic of this deficiency.

4. Rats living on a commercial chow were more resistant to the toxic effects of hyperthyroidism than animals on our best synthetic ration. The further addition of choline or methionine to this synthetic diet somewhat increased the susceptibility of rats to thyroid toxicity.

5. Animals on low protein diets were more susceptible to thyroid toxicity than those on high protein diets, and animals on high fat diets were somewhat more resistant than those on low fat diets. Animals deficient in both cystine and choline were more susceptible to hyperthyroidism than any other group studied.

6. The existence of an already established fatty liver appeared to protect the rat against thyrotoxicosis.

7. The inclusion of sulfasuxidine, taurine or inositol in a choline-deficient diet prevented the development of cirrhosis during the experimental period but not by virtue of lipotropic action or antithyroid activity, as judged by liver fat content and the histological appearance of the thyroid.

#### ACKNOWLEDGMENTS

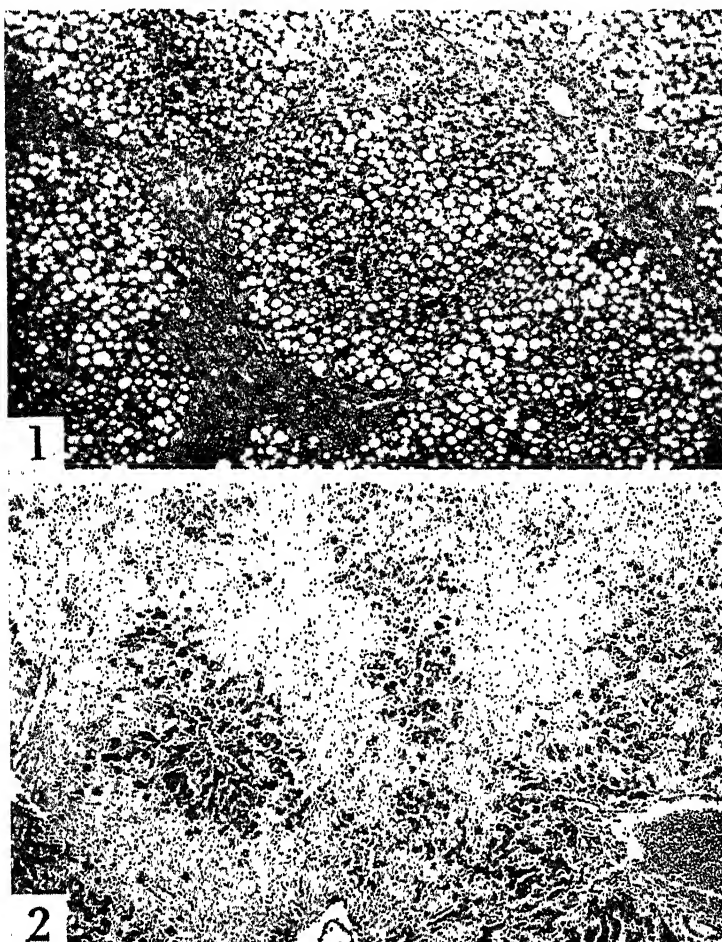
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1 Photomicrograph of liver from rat on basal choline-deficient diet. Note extreme fatty infiltration of liver cells which are present. Note also relatively large areas of scarring which replace hepatic parenchyma. Such scarred areas contain vacuoles of fat as well as refractile ceroid. These areas of necrosis and fibrosis tend to involve rather large areas. Typical nodular cirrhosis was not observed in these experiments.

2 Photomicrograph of liver from rat on basal cystine-deficient diet. Note absence of fatty infiltration of the few liver cells which remain. The majority of the hepatic cells are necrotic; only here and there a few viable looking cells can be seen. There is no scarring and very little cellular reaction.



# THE LYSINE, METHIONINE AND THREONINE CONTENT OF MEATS<sup>1</sup>

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The microbiological estimation of amino acids in meats was initiated in this laboratory by Schweigert, McIntire, Elvehjem and Strong ('44), and the contents of leucine, isoleucine and valine (Schweigert et al., '45), tryptophane (Greenhut et al., '46a, '47), threonine (Greenhut et al., '46b), and phenylalanine (Greenhut et al., '47) have been previously reported. The findings in this earlier work had indicated that meat proteins were strikingly similar with respect to their content of these 6 amino acids.

The methods employed in the previous work have been extended to the determination of methionine and lysine in meats, and to the assay of a greater variety of meat samples for their threonine content than were studied in the earlier work. In the present study *Leuconostoc mesenteroides* P-60 was used for the determination of methionine and lysine, while *Streptococcus faecalis* R was employed in the methods for methionine and threonine. The data obtained for the lysine, methionine and threonine content of meats have been subjected to a statistical analysis in an attempt to evaluate differences between cuts and the variation encountered in repeated analyses.

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## EXPERIMENTAL

*Preparation of samples*

Fresh cuts of beef, lamb, pork, veal and whole beef organs (table 2) were obtained at a local packing house, and were immediately prepared for analysis. Two of the cuts from each of the species represented were divided into uniform halves, and 1 portion from each of these was roasted according to methods described by McIntire et al. ('43). After the meats had been trimmed to remove bone, gristle and excess fat, they were passed through an electric food chopper 4 to 6 times and were mixed thoroughly to give homogeneous preparations. To insure the same moisture content in samples taken for nitrogen analysis and for hydrolysis, weighings were made simultaneously. Nitrogen determinations were carried out by a macro-Kjeldahl procedure. Hydrolysates were prepared by autoclaving 10-gm samples with 200 ml 2N HCl for 5 hours at 15 lbs. pressure in covered Erlenmeyer flasks. Preliminary studies had shown that increases in the concentration of the acid or the length of time of hydrolysis did not result in higher values for these 3 amino acids. The hydrolysates were filtered at room temperature, diluted to a convenient volume and stored at 5°C. Prior to each assay, portions of the hydrolysates were neutralized and further diluted to the appropriate concentrations required for each amino acid.

*Assay procedures*

The microbiological assay techniques described by Schweigert et al. ('44) have been applied with some modification to the determinations of lysine, methionine and threonine in fresh and cooked meats. To permit the simultaneous use of different organisms for the assay of several amino acids with 1 basal medium, the uniform medium developed by Henderson and Snell ('48) was adopted with respect to all constituents except for the levels of certain amino acids. These amino acids were incorporated at the levels which had been routinely

TABLE 1

*Basal medium for assays of lysine, methionine and threonine.<sup>1</sup>*

CONSTITUENT	AMOUNT/ 100 ML. FINAL CONCEN- TRATION	CONSTITUENT	AMOUNT/ 100 ML. FINAL CONCEN- TRATION
Glucose (gm)	2.0	<i>Amino acids</i>	
Na acetate (gm)	0.1	DL-alanine (mg)	20
Na citrate (gm)	2.0	L-arginine · HCl (mg)	10
NH <sub>4</sub> Cl (gm)	0.3	DL-aspartic acid (mg)	40
K <sub>2</sub> HPO <sub>4</sub> (gm)	0.5	L-cystine (mg)	20
<i>Salts C</i>		L-glutamic acid (mg)	40
MgSO <sub>4</sub> · 7H <sub>2</sub> O (mg)	80	Glycine (mg)	10
NaCl (mg)	4	L-histidine · HCl · H <sub>2</sub> O (mg)	10
FeSO <sub>4</sub> · 7H <sub>2</sub> O (mg)	4	DL-isoleucine (mg)	20
MnSO <sub>4</sub> · 7H <sub>2</sub> O (mg)	16	L-leucine <sup>2</sup> (mg)	10
Adenine sulphate (mg)	1	L-lysine · HCl · H <sub>2</sub> O (mg)	20
Guanine hydrochloride (mg)	1	DL-methionine (mg)	20
Uracil (mg)	1	DL-phenylalanine (mg)	10
Xanthine (mg)	1	L-proline (mg)	5
Thiamine (μg)	100	DL-serine (mg)	20
Riboflavin (μg)	100	DL-threonine (mg)	20
Pyridoxal (μg)	20	DL-tryptophane (mg)	10
Ca pantothenate (μg)	100	L-tyrosine (mg)	10
Niacin (μg)	100	DL-valine (mg)	20
p-Aminobenzoic acid (μg)	20		
Biotin (μg)	1		
Folic acid (μg)	1		

<sup>1</sup> Appropriate amino acid omitted from the medium.<sup>2</sup> DL-leucine substituted at 20 mg level for the methionine assays to avoid possible methionine contamination. (Riesen et al., '46.)

used in prior work, and the constituents of the medium as used are presented in table 1.

The assays were conducted on a semi-micro scale in which the final volume of medium per tube was 2 ml. In all cases the standard curves were based on at least 5 tubes at each of 6 different levels. The standard solutions were prepared to contain 18 μg of DL-threonine, 8 μg of L-methionine,<sup>2</sup> and 25 μg of L-lysine (added as the monohydrochloride). Samples were

<sup>2</sup> Kindly supplied by Dr. R. T. Major, of Merck and Co., Rahway, New Jersey.

TABLE 2  
*Analysis of meats.*

OUT OF MEAT	PROTEIN %	LYSINE			METHIONINE			THREONINE		
		No. of assays	In meat <sup>1</sup> %	Reten- tion <sup>2</sup> %	No. of assays	In meat %	Reten- tion %	No. of assays	In meat %	Reten- tion %
Veal										
Shank	21.7	8	1.73	8.0	5	0.43	2.0	3	0.95	4.4
Shoulder	20.4	8	1.63	8.0	5	0.47	2.3	4	0.86	4.2
Roast shoulder	27.4	6	2.09	8.0	6	0.63	2.3	5	1.15	4.2
Loin	17.1	6	1.48	8.3	7	0.39	2.3	4	0.77	4.5
Round	20.4	7	1.63	8.0	5	0.45	2.2	6	0.86	4.2
Roast round	29.0	4	2.52	8.7	5	0.67	2.3	4	1.30	4.5
Group mean			8.1				2.3			4.3
Lamb										
Shank	11.7	6	0.96	8.2	7	0.28	2.4	5	0.53	4.5
Roast shank	12.5	5	1.05	8.4	7	0.27	2.2	5	0.55	4.4
Shoulder	15.8	6	1.26	8.0	6	0.38	2.4	4	0.71	4.5
Loin	13.0	4	0.99	7.6	4	0.30	2.3	3	0.56	4.3
Leg	16.5	4	1.37	8.3	6	0.38	2.3	5	0.69	4.2
Roast leg	27.5	5	2.17	7.9	5	0.60	2.2	4	1.10	4.0
Group mean			8.1	96			2.3			4.3
Pork										
Picnic ham	16.5	6	1.32	8.0	6	0.36	2.2	7	0.69	4.2
Butt	12.2	8	0.96	7.9	6	0.27	2.2	2	0.54	4.4
Roast butt	22.8	6	1.94	8.5	7	0.50	2.2	6	1.00	4.4
Loin	17.4	3	1.38	7.9	8	0.40	2.3	5	0.76	4.4
Ham	13.9	6	1.15	8.3	4	0.35	2.5	3	0.64	4.6
Roast ham	27.6	2	2.10	7.6	5	0.64	2.3	3	1.27	4.6
Group mean				91			2.2			4.4
Beef										
Chuck	17.4	6	1.46	8.4	4	0.40	2.3	7	0.77	4.4
Roast chuck	22.7	5	1.84	8.1	5	0.50	2.2	6	0.95	4.2
Rib	16.7	3	1.32	7.9	4	0.37	2.2	5	0.73	4.4
				99			98			97

TABLE 2 (continued)  
*Analysis of meats.*

OUT OF MEAT	LYSINE				METHIONINE				THREONINE			
	PROTEIN	No. of assays	In meat <sup>1</sup>	In protein <sup>2</sup>	Reten- tion	No. of assays	In meat	In protein	Reten- tion	No. of assays	In meat	In protein
	%		%	%	%		%	%	%		%	%
Beef												
Loin end	17.6	4	1.35	7.7		5	0.39	2.2		4	0.74	4.2
Round	20.6	4	1.44	7.0		4	0.45	2.2		4	0.89	4.3
Roast round	33.4	3	2.94	8.8		3	0.80	2.4	114	4	1.47	4.4
Group mean				8.0				2.2				4.3
Brain	10.6	5	0.69	6.5		3	0.22	1.9		7	0.46	4.3
Brain	10.8	5	0.65	6.0		4	0.19	1.8		8	0.45	4.2
Brain	10.3	3	0.71	6.9		4	0.19	1.8		4	0.46	4.5
Mean				6.4				1.8				4.3
Heart	17.4	2	1.35	7.8		7	0.37	2.1		8	0.77	4.4
Heart	17.7	5	1.36	7.7		3	0.37	2.1		7	0.80	4.5
Mean				7.8				2.1				4.5
Kidney	16.7	5	1.13	6.8		3	0.28	1.7		6	0.73	4.4
Kidney	16.4	3	1.15	7.0		6	0.33	2.0		8	0.74	4.5
Kidney	16.0	5	1.07	6.7		6	0.29	1.8		3	0.69	4.3
Mean				6.8				1.9				4.4
Liver	21.0	5	1.47	7.0		3	0.50	2.4		6	0.88	4.2
Liver	20.8	4	1.45	7.0		3	0.48	2.3		6	0.90	4.3
Liver	20.4	4	1.43	7.0		4	0.45	2.2		3	0.90	4.4
Mean				7.0				2.3				4.3
Tongue	20.5	4	1.58	7.7		7	0.41	2.0		6	0.84	4.1
Tongue	20.2	6	1.53	7.5		6	0.40	2.0		7	0.89	4.4
Mean				7.6				2.0				4.3
Standard deviation				0.54				0.21				0.26
Coefficient of variability				7.0				9.6				6.0

<sup>1</sup> and <sup>2</sup> throughout signifies grams of amino acid per 100 gm of meat (or meat protein).



added in duplicate at each of 3 different levels, with all necessary measurements being made with either a Cannon dispenser<sup>3</sup> or the Cannon-Riesen aliquoter.<sup>4</sup> Assay tubes were sterilized by autoclaving at 15 lbs. for 15 minutes, and were inoculated with 1 drop per tube of a 25 ml sterile saline suspension of the test organism, centrifuged from 5 ml enriched medium in which growth had proceeded for 24 hours. The tubes were incubated at 37°C. for 72 hours, and the acid produced was titrated with the aid of a quinhydrone potentiometric titrator.<sup>4</sup>

### RESULTS AND DISCUSSION

The results of repeated assays for lysine, methionine and threonine in the various cuts of meats are presented in table 2. The amino acid content is expressed as the per cent found in the meat (grams amino acid per 100 gm of tissue) and as the per cent in the protein (calculated to 16% nitrogen). In the case of the cooked samples, the retention of these amino acids has been calculated on the basis of the total amount of each found in the whole cut before and after roasting. The number of assays on which the mean values are based is also indicated.

All data were treated statistically by the analysis of variance technique, as shown in table 3. A combined analysis of the data for each amino acid was used, since it was found that the mean squares within samples were homogeneous for each of the 3 amino acids. The *F* values obtained by this analysis indicate that there are significant differences in the lysine and methionine contents of the various meat samples, but no significant differences are found in the threonine contents. The specific location of these significant differences has been ascertained by means of the *t* test.

<sup>3</sup> To be published by M. D. Cannon.

<sup>4</sup> A complete description of the equipment being used in this laboratory for the preparation of microbiological assays will be published in a later paper.

TABLE 3

*Analysis of variance for data obtained in repeated assays for lysine, methionine and threonine in meats.*

SOURCE OF VARIATION	LYSINE			METHIONINE			THREONINE		
	Degrees of freedom	Mean square	F value	Degrees of freedom	Mean square	F value	Degrees of freedom	Mean square	F value
Between all samples	36	2.0178	6.84 <sup>1</sup>	36	0.1520	3.49 <sup>1</sup>	36	0.0860	1.25
Between cuts within veal	5	0.3977	1.35	5	0.0559	1.29	5	0.0733	1.06
Between cuts within lamb	5	0.3977	1.36	5	0.0286	< 1.00	5	0.1246	1.81
Between cuts within pork	5	0.4236	1.44	5	0.0669	1.54	5	0.0852	1.24
Between cuts within beef	5	1.3715	4.65 <sup>1</sup>	5	0.0160	< 1.00	5	0.0506	< 1.00
Between cuts within organs	8	0.2414	< 1.00	8	0.0324	< 1.00	8	0.1027	1.49
Between species for muscle tissues	3	0.0743	< 1.00	3	0.0399	< 1.00	3	0.0289	< 1.00
Between organs	4	3.2150	10.90 <sup>1</sup>	4	0.3675	8.45 <sup>1</sup>	4	0.1299	1.89
Between muscle tissues and organs	1	44.6630	151.45 <sup>1</sup>	1	2.7855	64.03 <sup>1</sup>	1	0.0002	< 1.00
Within samples	144	0.2949	...	151	0.0435	...	150	0.0689	...

<sup>1</sup> Indicates that F value exceeds 1% level of significance.

*Lysine*

Application of the *t* test to the lysine data indicates that the mean values of 6.4% in brain, 6.8% in kidney and 7.0% in liver are significantly lower than the mean of 8.0% lysine found in the protein of the beef muscle tissues, when considered at a 1% level of significance. Extending this test further, it can be shown that the mean for beef tongue is significantly lower than the group means for veal, lamb and pork muscle tissues, while the mean of 7.8% lysine in heart proteins is no different from that of the other muscle tissues. No such differences can be detected within the cuts of the various muscle tissues, except in the case of round and roast round of beef. This latter difference appears to be significant, but since the values are based on only 1 preparation of hydrolysate and on only 4 and 3 assays, respectively, it is felt that no practical significance can be attached to the difference. However, it is possible that the difference may be peculiar to this particular sample of meat or to the preparation thereof.

Microbiological methods for the determination of lysine have been reported by many workers (Dunn et al., '44; Hier et al., '45; Stokes et al., '45; Guirard et al., '46), but data regarding the lysine content of meats and meat proteins are limited. Hier et al. ('45) reported 7.9% in beef muscle protein, and Stokes et al. ('45) found 6.1% in beef liver, both workers having used *S. faecalis* as the assay organism. These values compare favorably with the 8.0% lysine in beef muscle tissues and the 7.0% in beef liver found in the present work by use of the same organism. Beach et al. ('43), employing a chemical procedure for the analysis of lysine, found 8.11, 9.62, 8.68 and 8.65% in the muscle tissue proteins of beef, veal, lamb and pork, and 7.10, 6.02, 6.21 and 5.98% in beef heart, liver, kidney and brain, respectively. These workers pointed out that one of the outstanding differences between organ and muscle tissues found in their analyses of 10 different amino acids was the lower level of lysine in organs. The present work serves to give statistical verification to this difference,

though the average differences appear to be considerably smaller than those found by Beach et al.

### *Methionine*

The data in table 2 show that the proteins of the muscle tissues and of beef liver average 2.2 to 2.3% in their content of methionine, with no significant differences discernible among any of these groups. Values obtained for beef brain, kidney and tongue are 1.8, 1.9 and 2.0%, all of which are significantly lower than those for the muscle tissue groups. The 2.1% value found for beef heart appears to be significantly lower than the 2.3% found in veal and lamb muscle tissues, but is higher than the 1.8 and 1.9% methionine found in beef brain and kidney proteins.

Earlier work reported from this laboratory by Riesen et al. ('46) indicated a considerably higher content of methionine in some of the meat tissues. Three different organisms, *L. arabinosus*, *Leuc. mesenteroides* and *S. faecalis*, gave essentially the same values under the earlier conditions, and some of the values reported included 3.03% methionine in the proteins of pork muscle, 3.09% in beef heart, 1.93% in beef kidney and 2.55% in beef brain. In the present study, both *S. faecalis* and *Leuc. mesenteroides* were used interchangeably with excellent agreement, though all results were consistently lower than previously reported.

A comprehensive study of the methionine content of meats has been conducted by Lyman et al. ('46a), who reported values ranging from 2.09% in beef kidney protein to 2.50% in the protein of beef round. Though in general their values are somewhat higher than those found in this present study, essentially the same relative differences were found between muscle tissues and organs, with the exception of beef liver, which they found to be almost as low in methionine as some of the other organs, e.g., beef tongue. Other values found in the literature for the methionine content of meats include 2.0% in beef liver (Stokes et al., '45), 2.75% in beef muscle

(Riesen et al., '46), 3.06 to 3.62% in various muscle tissues (Beach et al., '43) and 4.12% in beef round (Edwards et al., '46). The 2 former groups of workers used a microbiological technique; the latter groups employed chemical methods in their work. In general, values obtained by chemical methods appear to be considerably higher than those found by microbiological means, though Lyman et al. ('46b) were able to obtain excellent agreement between 2 different microbiological methods and a colorimetric method.

### *Threonine*

Although the proteins of various meat muscle tissues and organs show significant differences in lysine and methionine content, they are remarkably similar in threonine content. No statistically significant difference could be detected among any of the various groups of meats, with individual cuts and organs averaging 4.0 to 4.6% in the proteins, giving an over-all average of 4.3%.

In a previous publication by Greenhut et al. ('46b) from this laboratory on the determination of threonine in natural products with *S. faecalis*, values were reported on several different meat tissues. A rather high degree of variation had been encountered, and values ranged from 3.7 to 4.7% threonine in the proteins. Some of this variation may be attributed to the fact that the assays had been conducted with samples which had been stored at  $-5^{\circ}\text{C}$ . for 2 to 3 years, with brief partial thawing at intervals of 3 to 4 months. These conditions may have been responsible for destruction of some threonine, or may have led to nitrogen losses which would account for high values obtained in calculating to a 16% nitrogen basis. In general, however, the present results are in good agreement with the earlier work.

Other workers have reported 5.4% threonine in beef muscle proteins (Hier et al., '45), 3.8% in beef liver proteins (Stokes et al., '45) and 3.34 to 3.92% in various muscle tissues and beef organs (Beach et al., '43).

*Standard deviation*

The standard deviations for lysine, methionine and threonine, as shown in table 2, are 0.54, 0.21 and 0.26, respectively, with reference to the per cent of amino acid found in the meat proteins. The coefficient of variability, which expresses the standard deviation in per cent of the arithmetic mean, was 7.0% for lysine, 9.6% for methionine and 6.0% for threonine. Both of these statistical entities are measures of the random, uncontrolled variation found in repeated assays, and as such serve as a guide to the degree of confidence that can be placed on values determined from a limited number of assays.

The values presented in table 2 for the retention of these amino acids indicate that there is no significant reduction in their total amounts during the cooking process. There is, however, a wide range in the amount of protein found in the different meats: Pork and lamb cuts tend to have less protein than the beef and veal, reflecting a higher content of fat in the former. From a nutritional standpoint, these wide differences in protein content are of importance in the evaluation of total amino acid intake, even though the meat proteins themselves appear to be quite similar.

## SUMMARY

*Leuconostoc mesenteroides* was used for the determination of lysine and methionine in meats. *Streptococcus faecalis* was used for similar determinations of methionine and threonine. The analyses were made on acid hydrolysates of the crude homogeneous meat samples. The average threonine content of 24 different skeletal muscle tissues was 4.4% of the crude protein, while the lysine content was 8.1% and the methionine content was 2.3%. A statistical analysis indicated that no significant differences existed between the results obtained for the muscle tissue proteins, but that beef brain, kidney and tongue were significantly lower in methionine while beef brain, kidney and liver were significantly lower in lysine. Studies on

retention show that these amino acids are not lost or destroyed in meats during the cooking process.

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# RELATIVE SIGNIFICANCE OF GROWTH AND METABOLIC RATE UPON THE UTILIZATION OF VITAMIN A BY THE RAT<sup>1</sup>

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Recent experiments indicate the importance of thyroxine in the conversion of carotene to vitamin A in the rat (Johnson and Baumann, '47b), but it is not entirely clear whether the requirement for the vitamin itself increases with increased metabolic rate. Claims to the latter effect have been frequent (Abelin, '33; v. Euler and Klusmann, '32; Schneider and Widmann, '34; Wendt, '35; Greaves and Schmidt, '36; Sure and Buchanan, '37), although contradictory data have also been recorded. When groups of normal and hyperthyroid rats containing uniform stores of vitamin A were fed a diet low in vitamin A, the amounts of this factor remaining in the liver were usually at least as high in the hyperthyroid group as in the controls (Logaras and Drummond, '38; Baumann and Moore, '39), and in some series the administration of thyroxine actually appeared to decrease the rate of utilization of vitamin A. Hyperthyroid rats fed moderate amounts of fish liver oil stored approximately as much vitamin A as control rats

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(Johnson and Baumann, '47b). However, factors that alter the metabolic rate usually also alter the rate of growth, and a large animal might reasonably be expected to require more vitamin than a small one. The present experiments were therefore designed to evaluate the relative significance of the 2 factors, growth and metabolic rate, upon the metabolism of vitamin A in the rat.

#### METHODS

The experiments were performed on series of comparable weanling rats either from our own stock colony or from the Sprague-Dawley Company; rats from the 2 sources proved to be quite similar. The groups within each series were comparable in weight, age and sex. At the beginning of the experiment, each rat was fed 6 drops (approximately 0.12 gm) of a vitamin A concentrate prepared by mixing 1 gm of halibut liver oil (approximately 60,000 I.U. per gm) with 3 gm of cottonseed<sup>3</sup> oil. Three drops of the mixture were fed in the morning and the other 3 drops, 8 hours later. This was sufficient to produce hepatic stores of approximately 250  $\mu$ g of vitamin A, as determined by analyses made on animals killed 72 hours after the administration of the second portion of the concentrate. The animals were then maintained on various diets low in vitamin A and subjected to the conditions indicated below in order to promote different metabolic rates or different rates of growth.

The basal diet had a percentage composition of vitamin A-free casein 18, dextrin 65, cottonseed<sup>3</sup> oil 5, brewers' yeast 8, and salts (Wesson, '32) 4. Sixteen drops of a propylene glycol solution<sup>4</sup> containing 10,000 U.S.P. units per gram of a crystalline vitamin D from ergosterol were added per 10 kg of diet. Decreased growth rates were effected either by restricting the caloric intake to  $\frac{1}{2}$  or  $\frac{3}{4}$  of that consumed by the control animals, or by feeding a diet low in either thiamine or trypto-

<sup>3</sup> Wesson.

<sup>4</sup> Drisdol, containing 400 units of vitamin D, Winthrop Chemical Company, Inc., New York, N. Y.

phane. In the diet low in thiamine, brewers' yeast autoclaved for 4 hours at 15 pounds pressure replaced the ordinary yeast in the low A diet. As the depletion period was prolonged, enough thiamine hydrochloride was fed by dropper to enable the rats of this group to maintain weight. In the diet low in tryptophane, the casein of the basal ration was replaced by an equivalent amount of a casein hydrolysate prepared according to the method of Woolley ('46). Since this ration contained 8% of yeast, there was enough tryptophane present to permit the animals to maintain weight for 2 months with slight growth during the first few weeks. A relatively rapid rate of growth, 4 gm/rat/day during the depletion period, was produced in 1 group by the substitution of 0.5% of a solubilized whole liver preparation<sup>5</sup> for an equivalent amount of brewers' yeast; comparable rats on the control diet gained 3.3 gm/day.

The basal metabolic rates of the rats were decreased by feeding either thiourea incorporated into the basal diet at a level of 0.5%, or 2-thiouracil administered in the drinking water at a level of 0.1%. Increases in metabolic rates were produced by feeding desiccated thyroid at a level of 1 mg per gram of body weight per day. This material was suspended in water at a concentration that supplied the required amount in 3 drops of the suspension. It was prepared fresh every 3 days, and was kept stored in a refrigerator. In 1 experiment, 2,4-dinitrophenol was used to increase the metabolic rates in rats fed the basal low A diet. The compound was dissolved in dilute aqueous  $\text{NaHCO}_3$  solution at a concentration of 4 mg per milliliter, and the solution was injected subcutaneously at a level of 40 mg per kilogram of body weight per day. The daily dose was injected in 2 equal portions, given 8 hours apart. Quantitative data on the consumption of oxygen by the rats were obtained in 1 series by means of an apparatus modified from that described by Schwabe and Griffith ('38).

After suitable intervals of time, selected to permit the vitamin A content of the livers to decrease measurably, the rats were killed by decapitation, and the livers and kidneys were

<sup>5</sup> Lederle liver preparation no. 1432, found to contain 1.8  $\mu\text{g}$  vitamin A per gram.

removed and analyzed individually for vitamin A by the procedure described previously (Johnson and Baumann, '47a). Analyses were either made periodically, after 15, 30 and 60 days, or in the later series, after 45 days of depletion.

#### EXPERIMENTAL

##### *Depletion of vitamin A reserves in growing and non-growing rats*

In line with the observations of others, rats lost vitamin A from the liver when fed diets deficient in this vitamin for long periods of time. In a typical series (A, table 1) in which the basal low A diet was fed *ad libitum*, the liver stores decreased from an initial value of 228  $\mu\text{g}$  at the beginning of the experiment to 42  $\mu\text{g}$  60 days later. In a second series, B, the values declined from an initial 238  $\mu\text{g}$  to 41  $\mu\text{g}$  after 45 days. In both series the rats grew well, an average of 208 gm in 60 days in series A, and an average of 175 gm in 45 days in series B. However, when the growth of the rats was hindered by feeding an inadequate amount of food, or by feeding diets that were deficient in tryptophane or in thiamine, the amounts of vitamin A that remained in the liver were appreciably higher than in rats that grew well. Rats fed only  $\frac{1}{2}$  the amount of food consumed by the control group gained only 33 gm in 60 days in series A, and 33 gm in 45 days in series B (table 1). The amounts of hepatic vitamin A remaining at the end of the experiment were 111  $\mu\text{g}$  and 188  $\mu\text{g}$  in the 2 series, respectively, as compared to values of 42 and 41  $\mu\text{g}$  in the control groups at these times. Thus, the animals that grew well lost 2-4 times as much of the original hepatic vitamin A as did the partially starved rats. Essentially similar results were observed when the growth rate was decreased by feeding diets inadequate in tryptophane or in thiamine. The low-tryptophane rats grew only 33 gm in 60 days, and contained 91  $\mu\text{g}$  of vitamin A, as compared with 42  $\mu\text{g}$  found in the normally growing control animals. A diet low in thiamine resulted in a growth of 43 gm in 45 days, with 108  $\mu\text{g}$  of vitamin A remaining in the liver,

as compared with 41  $\mu\text{g}$  found in the control animals that gained 175 gm.

TABLE 1

*The influence of body size upon the depletion of vitamin A from the liver.<sup>1</sup>*

DIET	FEEDING PERIOD	BODY WT. GAINS	VITAMIN A			
			Liver	Kidney	Per gm of kidney	Total: kidney + liver
	days	gm	$\mu\text{g}$	$\mu\text{g}$	$\mu\text{g}$	$\mu\text{g}$
<i>Series A</i>						
Initial stores	3	..	228	1	1.8	229 (206-261)
Basal, <i>ad lib.</i>	30	104	156	18	13.8	174 (143-216)
Basal, <i>ad lib.</i>	60	208	42	5	3.5	47 ( 33- 59)
Basal, $\frac{1}{2}$ caloric intake	30	30	195	1	1.5	196 (191-229)
	60	33	111	1	1.1	112 (111-123)
Low tryptophane	15	25	225	1	1.3	226 (175-232)
Low tryptophane	30	36	145	1	1.7	146 (128-157)
Low tryptophane	60	33	91	1	1.2	92 ( 45-123)
<i>Series B</i>						
Initial stores	3	..	238	3	3.9	241 (197-259)
Basal, <i>ad lib.</i>	30	136	126	12	9.3	138 (109-185)
Basal, <i>ad lib.</i>	45	175	41	29	16	70 ( 52- 87)
Basal, $\frac{1}{2}$ caloric intake	30	28	205	2	3.3	207 (192-232)
	45	33	188	2	2.1	190 (172-222)
Thyroid, <sup>2</sup> $\frac{1}{2}$ caloric intake	30	— 5	186	1	1.4	187 (177-214)
	45	5	172	2	1.6	174 (167-180)
Low thiamine	30	34	179	2	2.4	181 (138-221)
Low thiamine	45	43	108	3	3.0	111 ( 93-126)

<sup>1</sup> Each figure represents the mean of 4 animals.

<sup>2</sup> Thyroid fed at a level of 1 mg per gm of body wt. per day.

### *Effect of metabolic rate upon vitamin A retention*

Thiourea and thiouracil in doses known to lower the basal metabolic rate by 20 to 35% (Reineke et al., '45; Christensen, '45), markedly delayed the disappearance of vitamin A from the liver. In series A (table 2) rats fed the low A diet plus 0.5% thiourea still retained 186  $\mu\text{g}$  of hepatic vitamin A out of an original 282  $\mu\text{g}$  after 60 days of depletion, as compared to

only 23  $\mu\text{g}$  remaining in the control groups. Expressed as microgram of vitamin A lost from the liver, the controls lost 126  $\mu\text{g}$  in 30 days, and 259  $\mu\text{g}$  in 60 days, whereas those receiving thiourea lost only 20 and 96  $\mu\text{g}$  at these times, respectively. Rats receiving 0.1% thiouracil in the drinking water (table 2,

TABLE 2

*The influence of basal metabolic rate upon the depletion of vitamin A from the liver.<sup>1</sup>*

DIET	FEEDING PERIOD	BODY WT. GAINS	VITAMIN A			
			Liver	Kidney	Per gm of kidney	Total: kidney + liver
	days	gm	$\mu\text{g}$	$\mu\text{g}$	$\mu\text{g}$	$\mu\text{g}$
<i>Series A</i>						
Initial stores	3	. .	282	2	3.3	284 (260-320)
Basal, <i>ad lib.</i>	30	104	156	11	8.4	167 (129-194)
Basal, <i>ad lib.</i>	60	210	23	23	11.8	46 ( 30- 64)
Basal + thiourea <sup>2</sup>	30	38	262	0.4	0.57	262 (241-287)
Basal + thiourea <sup>2</sup>	60	59	186	0.4	0.64	186 (164-216)
Basal + thyroid <sup>3</sup>	30	59	171	3	2.0	174 (159-185)
<i>Series B</i>						
Initial stores	3	.	265	2	2.6	267 (234-294)
	45	171	76	8	4.7	84 ( 40-109)
Basal, $\frac{1}{2}$ caloric intake + thyroid <sup>3</sup>	45	8	149	1	1.0	150 (115-212)
Basal + thyroid <sup>3</sup>	45	111	48	5	2.6	53 ( 41- 66)
Basal + thiouracil <sup>4</sup>	45	131	131	3	1.8	134 (121-140)

<sup>1</sup> Each figure represents the mean of 4 animals.

<sup>2</sup> Thiourea incorporated into the basal diet at a level of 0.5%.

<sup>3</sup> Thyroid fed at a level of 1 mg per gm of body wt. per day.

<sup>4</sup> 2-Thiouracil administered at a level of 0.1% in drinking water.

series B) likewise retained more hepatic vitamin A than control rats; after 45 days of depletion, the average amounts retained were 131  $\mu\text{g}$  and 76  $\mu\text{g}$ , respectively. Since, however, the thio-compounds had also retarded the rate of growth, e.g., the rats fed thiourea grew only 59 gm in 60 days as compared

to a gain of 210 gm by the control groups (table 2), the observed differences in vitamin retention could not be ascribed solely to the altered metabolic rate. Indeed, the effects of the thiourea, both on the reduction in growth rate and on increased vitamin retention (table 2, series A), were of the same order of magnitude as those previously observed when the intake of food was restricted by 50% (table 1).

Nevertheless, an effect on vitamin A by the basal metabolic rate as distinct from body size or the growth process is suggested in table 2, series B. Rats fed 1 mg of thyroid per gram of body weight grew 111 gm in a depletion period of 45 days, as compared to 131 gm by a comparable group receiving thiouracil, yet in spite of this similarity in growth the rats receiving thyroid lost their reserves of vitamin A much faster than those receiving thiouracil, the amounts of vitamin remaining in the liver being 48 and 131  $\mu$ g in the 2 groups, respectively.

In an attempt to determine the relative significance of growth and metabolic rates on the disappearance of vitamin A, rats with uniform stores of the vitamin were fed 3 levels of food, the amount consumed *ad libitum*, or  $\frac{3}{4}$  or  $\frac{1}{2}$  of this amount. At each level of intake, 1 group received the basal low A diet, another the diet plus supplements of desiccated thyroid, while a third received thiouracil in the drinking water. Rats on the control diet *ad libitum* gained 148 gm in 45 days. All other groups gained lesser amounts, the least being a gain of 9 gm by the low calorie group receiving thyroid (table 3). The amounts of vitamin A present in the liver after 45 days definitely indicated the importance both of the metabolic rate and of body size for the utilization of this vitamin. Normal rats whose caloric intakes were restricted by 25% were approximately as big as full-fed rats receiving either thyroid or thiouracil, the gains in 45 days being 82, 89 and 81 gm, respectively. In spite of this similarity in size, the amounts of vitamin A retained varied inversely with the metabolic rate. The rats on thiouracil, with an  $O_2$  consumption 26% below normal contained 229  $\mu$ g of vitamin A, a loss of only 61  $\mu$ g from the



TABLE 3

*The relative significance of body size and basal metabolic rate on vitamin A retention after 45 days.<sup>1</sup>*

DIET	BODY WT. GAINS	VITAMIN A			OILANGE IN O <sub>2</sub> CON- SUMPTION
		Liver	Kidney	Total: kidney + liver	
	gm	μg	μg	μg	%
Initial stores	..	290	2	292 (284-305)	..
Low A, <i>ad lib.</i>	148	136	33	169 (116-197)	0
Low A, $\frac{3}{4}$ caloric intake	82	182	8	190 (170-246)	- 11
Low A, $\frac{1}{2}$ caloric intake	49	187	3	190 (185-231)	+ 9
Low A + thyroid, <sup>2</sup> <i>ad lib.</i>	89	127	5	132 ( 74-175)	+ 220
Low A + thyroid, <sup>2</sup> $\frac{3}{4}$ caloric intake	42	190	3	193 (160-214)	..
Low A + thyroid, <sup>2</sup> $\frac{1}{2}$ caloric intake	9	212	3	215 (133-232)	+ 238
Low A + thiouracil, <sup>3</sup> <i>ad lib.</i>	81	229	2	231 (187-297)	- 26
Low A + thiouracil, <sup>3</sup> $\frac{3}{4}$ caloric intake	83	162	3	165 (152-214)	- 25
Low A + thiouracil, <sup>3</sup> $\frac{1}{2}$ caloric intake	42	247	2	249 (201-289)	- 25.5
Low A + Dinitrophenol <sup>4</sup>	125	144	5	149 (126-189)	+ 94
Solubilized liver preparation <sup>5</sup>	179	113	28	141 (100-193)	- 6

<sup>1</sup> Each figure represents the mean of 5 animals.

<sup>2</sup> Thyroid fed at a level of 1 mg per gm of body wt. per day.

<sup>3</sup> 2-Thiouracil administered at a level of 0.1% in drinking water.

<sup>4</sup> 2,4-Dinitrophenol given in 2 daily injections at a level of 20 mg of the drug per kg of body wt. per injection.

<sup>5</sup> Lederle's liver preparation no. 1432, incorporated into the basal low A diet at a level of 0.5%.

original stores of 290 μg (table 3). The normal rats on mild caloric restriction retained 182 μg of vitamin A, a loss of 108 μg, while rats of similar size fed thyroid retained only 127 μg, a loss of 163 μg. The latter rats had a metabolic rate 220% above normal.

In agreement with the results cited in the introduction, desiccated thyroid appeared to have variable effects on vita-

min A retention. In some series (table 2, series B; table 3, groups fed *ad libitum*), the hyperthyroid rats retained less vitamin A than faster-growing rats in the corresponding control groups. This result emphasizes the fact that an increased metabolic rate *per se* may increase the rate of utilization of vitamin A. In other series (table 2, series A; table 3, series receiving  $\frac{1}{2}$  or  $\frac{3}{4}$  caloric intake) the rats receiving thyroid actually retained more vitamin A than control rats not receiving thyroid, presumably because the hyperthyroid rats grew only about  $\frac{1}{2}$  as well as their respective controls. Since the dose of thyroid employed was sufficient to increase the metabolic rate by 220–238%, the fact that the hyperthyroid rats contained more vitamin A than their controls leads to the conclusion that a decrease of 50% in the growth rate can have a greater quantitative significance on the retention of hepatic vitamin A than a 3-fold increase in the metabolic rate.

The administration of 2,4-dinitrophenol increased the metabolic rate by 94% without any comparable effect either on the growth rate or on the retention of vitamin A. The average growth in the group receiving dinitrophenol was 125 gm in 45 days as compared to 148 gm in the control group, and the retention of vitamin A was essentially the same in the 2 groups, 144 and 136  $\mu$ g, respectively. In other words, a decrease of 23 gm in body size preserved more vitamin A than that lost because of a 94% increase in metabolic rate. The addition of a soluble liver extract to the basal low A diet resulted in a slightly decreased retention of vitamin A. Rats fed the liver extract gained an average of 179 gm in 45 days as compared to 148 gm gained by the control group. Hepatic stores of the vitamin at this time averaged 113  $\mu$ g in the faster-growing group as compared to 136  $\mu$ g in the controls (table 3).

#### *Translocation of vitamin A from liver to kidney*

In a previous study an increase in the vitamin A content of the kidneys was observed in rats that were rapidly losing hepatic vitamin A under the influence of dibenzanthracene

(Baumann et al., '42). The present experiments indicate that this shift of vitamin A to the kidneys is not necessarily dependent upon the presence of dibenzanthracene, but also occurs in normal rats depleted of vitamin A. An increase was observed in both the amount and the concentration of vitamin A in the kidneys of all rats that grew appreciably during the depletion period (tables 1, 2 and 3); and in a few groups (table 1, series B; table 2, series A) the amount of vitamin A in the kidney approached that in the liver as the depletion period was prolonged. However, no comparable increase in the concentration of vitamin A occurred in kidneys from rats whose growth had been restricted during the depletion period, and the amounts were particularly low in the kidneys of rats exposed to thiourea.

#### DISCUSSION

The need for certain of the B vitamins is generally regarded as depending directly and almost proportionally on the metabolic rate (Cowgill and Palmieri, '33; Drill, '38; Drill and Overman, '42). The present experiments indicate that the depletion of vitamin A from the liver depends upon metabolic rate to some extent, but that body size may affect vitamin A reserves to a greater extent than metabolic rate. In certain other respects, however, the retention of vitamin A in the liver seemed to parallel that of the B vitamins more closely. It has been observed that a reduction in food intake increases the concentration of riboflavin in the liver (Griffin and Baumann, '46), as well as that of most other B vitamins (Wright and Skeggs, '46), and the present data indicate that the retention of vitamin A is similarly improved when the intake of food is restricted. Another point of similarity between vitamin A and the B vitamins lies in the adverse effect on vitamin storage of proteins deficient in certain essential amino acids. Diets low in tryptophane impair the storage of riboflavin and pyridoxine<sup>6</sup> and the present experiments indicate in a preliminary way that this may also be true of vitamin A. Rats on

<sup>6</sup> Sauberlich and Griffin, unpublished results.

the diet low in tryptophane retained less vitamin A than those of comparable size fed a good protein (casein in the low A diet) but an insufficient amount of food (table 1, series A). After 30 days of depletion, the low calorie rats retained 196  $\mu$ g of an original 229  $\mu$ g of vitamin A, as compared to 146  $\mu$ g on the diet low in tryptophane. The losses of vitamin A were therefore 33 and 83  $\mu$ g of the vitamin on the 2 diets, respectively.

The present experiments furnish some basis for evaluating a previous conclusion that thyroid enhances the conversion of carotene into vitamin A (Johnson and Baumann, '47b). Normal rats fed 40  $\mu$ g of carotene daily for 2 weeks stored an average of 52.3  $\mu$ g of vitamin A in the livers and kidneys in contrast to an average storage of 92.5  $\mu$ g by hyperthyroid rats receiving similar amounts of carotene. The 2 groups of rats were not significantly different in size. Since it is now clear that the need for vitamin A increases somewhat when the basal rate goes up, it follows that the true effect of the thyroid principle upon the conversion of carotene to vitamin A must have been even greater than that indicated by the differences in vitamin storage observed.

#### SUMMARY

1. Rats whose growth was restricted by diets inadequate in calories, thiamine or tryptophane retained more hepatic vitamin A than control rats that grew normally.

2. Comparisons between rats of similar size suggested that metabolic rate also influenced vitamin A depletion; desiccated thyroid hastened the depletion of vitamin A reserves somewhat, while thiourea and thiouracil delayed it. Quantitatively, the effect of a 3-fold increase in metabolic rate was less important for vitamin A retention than a decrease in the growth rate by 50%.

3. In normally growing rats a decrease in the hepatic reserves of vitamin A was accompanied by an increase in the amount and concentration of the vitamin in the kidney. No such increase occurred in the kidneys of rats whose growth

was restricted during the depletion period. The concentration of vitamin A in the kidney was particularly low in rats exposed to thiourea.

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# IRON AND COPPER METABOLISM OF YOUNG WOMEN ON SELF-SELECTED DIETS<sup>1</sup>

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Few copper metabolism studies have been made with adults on self-selected diets. Two of these have been reported by Leverton ('39) and Leverton and Binkley ('44). In the earlier study 24 1-week metabolism studies which involved the weighing and analysis of all the excreta were made of young women between the ages of 16 and 25 years on self-chosen diets. Results indicated that there was a relation between the level of copper intake and the amount of copper stored. Whenever the intake was less than 2 mg there was a negative balance. From this study it was concluded that a maintenance allowance of 2 mg of copper should be allowed girls between the ages of 16 and 25 years; if more is present in the diet, it will be stored.

In the later study copper metabolism experiments covering 95 1-week periods were made on 65 young women on self-chosen diets. Also, a long-term study was made on 4 young women on an adequate constant diet. The results were similar to those of the first study, i.e., as the copper intake increased, a large proportion of each increase was retained. It was also concluded that a daily allowance of 2.0 to 2.5 mg of copper is adequate and that this amount can be obtained from diets of medium nutritive value.

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A daily iron requirement of 12 mg for young adults is an accepted recommendation.

Because of the frequent occurrence of nutritional anemia in this age group, it seemed desirable to determine both the copper and the iron intake and output on self-selected diets.

#### PROCEDURE

The subjects used in this study were young college women between the ages of 17 and 22 years who were living in the Home Management House Duplex, North Texas State College, at the time of their participation in the study. All food, feces and urine were analyzed. A serving of each food from each meal, similar in all respects to that eaten by the college women, was collected at the same time that the subjects were served at the table. One-fourth of a glass of milk was collected daily in a similar manner, thus giving 1 homogeneous milk sample for analysis. A record was kept of the number of glasses of milk consumed by each girl each day since this was the only variable in the daily food intake. Suitable additions were then made to each individual's food intake to include the milk. After weighing the food to get the total weight, the food was macerated in the Waring Blendor, and a known fraction of the weight was removed into 1 common jar and kept in the refrigerator until analysis could be made. In some instances meals were eaten outside the house. In this case duplicates of the foods eaten were collected and analyzed separately. Appropriate corrections were made for that individual's food intake for the day.

Carminc was given to mark the feces. After weighing, the feces were macerated in the Waring Blendor and refrigerated. The urine was collected directly in 4-liter amber bottles containing a preservative.

For the calibration curve a standard iron solution was prepared by dissolving 1 gm of electrolytic iron in 1 liter of 10% sulfuric acid (1 mg of iron per milliliter). The Cenco-Sheard-Sanford photometer was set at 100 with a blank

solution and readings were taken on the standard solutions, using a 515 mμ filter.

The calibration curve for copper was prepared in the same manner, using a standard copper solution in the place of the standard iron solution. The standard copper solution was made by weighing out 3.93 gm of copper sulfate crystals ( $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$ ), dissolving in water and diluting to 1 liter (1 mg of copper per milliliter). The readings were also made on a Cenco-Sheard-Sanford photometer, using a 515 mμ filter.

Recoveries ranged from 93 to 106% for iron and 92 to 108% for copper.

From the macerated food of known weight, a 15-gm sample, plus allowance for the 1% metaphosphoric acid, was taken for analysis. The sample was weighed into a large porcelain crucible, 10 ml of 50% ammonium acetate solution was added, and it was evaporated to dryness and ashed in the electric muffle at a temperature not exceeding 500°C. (Stone, '42).

Simultaneous determinations of iron and copper were made, using Parker and Griffin's ('39) method of combining the dipyridyl for the estimation of available iron with McFarlane's ('32) carbamate method for the determination of copper in organic materials.

Liter samples of tap water were ashed and also analyzed for iron and copper. There was no detectable iron or copper in 1 liter of tap water. Consequently, the amount of water consumed by these subjects was not important from the standpoint of iron and copper intakes.

#### DISCUSSION

Because of the close nutritional relationship between iron and copper, both elements were determined in the food, feces and urine of 17 young college women. The girls in each group had the same self-chosen diet with a few exceptions. For instance, M.J.C., L.D.W. and M.G.M. (study I-A) were in the first 5-day study. M.J.C. and L.D.W. had the same self-chosen diet as M.G.M., with the exception of 2 lunches which

they had away from the house. While these 2 lunches did not give the same iron and copper values as the lunches served in the house, they did not materially affect the average daily intake.

Due to unavoidable conditions, only one subject (L.H., study I-B) was on the diet for the whole 5-day period. The self-selected diets varied greatly in the daily iron and copper contents; in fact, so much so, that the 2 girls, P.A. and M.J., who participated on the first 3 days of the period, had much lower daily average intakes than G.R., who was on the diet the last 3 days. The fact that L.H. was on the diet for the full 5 days also gave her lower iron and copper daily average intakes than G.R.

The other variation in the intakes of iron and copper was caused by the different amounts of milk consumed by each of the subjects. Both the first and second studies, covering 5 and 4 days, respectively, included the variable milk consumption of the individual girls. The daily average milk intake was from 0 to  $3\frac{1}{2}$  cups. This milk contributed from 0.0 to 1.7 mg of iron and from 0.0 to 2.9 mg of copper to the total daily intakes of these 2 minerals.

The lowest iron intake was observed in study II-A, followed by study I-A and study II-B, with the largest iron intake in study I-B, table 1.

The copper intake did not follow the same sequence of increase which occurred with the iron. In general, the lowest intake of copper was found in the study I-A group, with both groups of study II showing slightly larger amounts consumed. L.H. and G.R. from study I-B, and E.R. of study II-B had the highest average daily intakes of copper. The average daily intakes of iron and copper in food and milk were 6.6 to 13.6 mg with an average of 9.6, and 6.5 to 13.0 with an average of 8.1 mg, respectively.

Leverton and Binkley ('44) found that average daily intakes of 10.4 and 2.7 mg for iron and copper, respectively, on self-selected diets maintained a positive balance for their subjects. The daily average intakes in the present study

TABLE 1  
*Iron and copper content of food, urine and feces with calculated retention.*

NAME	DAYS	INTAKE				EXCRETION				RETENTION			
		Food plus milk		Urine		Feces		Total		Iron		Copper	
		Total		Total		Total		Total		Total		Average	
		Iron	Copper	Iron	Copper	Iron	Copper	Iron	Copper	Total	Average	Total	Average
		mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg
M.G.M.	5	44.8	40.5	0.2	0.1	Study I-A		5.9	3.7	6.1	3.8	38.7	7.7
M.J.C.	5	37.7	32.6	1.7	0.4	7.0	2.9	8.7	3.3	29.0	5.8	29.3	5.9
L.D.W.	5	41.7	36.3	1.7	0.9	9.5	5.7	11.2	6.6	30.5	6.1	29.7	5.9
P.A.	3	35.3	24.2	0.4	0.3	Study I-B		3.5	2.0	3.9	2.3	31.4	10.5
G.R.	3	40.8	38.9	0.3	0.4	3.7	1.9	4.0	2.3	36.8	12.3	36.6	12.2
L.H.	5	60.4	49.8	0.3	0.4	6.4	1.7	6.7	2.1	53.7	10.7	47.7	9.5
M.J.	3	35.3	24.2	0.6	1.1	5.5	4.0	6.1	5.1	29.2	9.7	19.1	6.4
M.H.B.	4	26.6	30.6	1.6	1.3	Study II-A		3.5	0.9	5.1	2.1	21.5	5.4
F.D.	4	26.4	30.4	2.0	1.6	2.9	3.1	4.9	4.7	21.5	5.4	25.7	6.4
M.R.	4	27.9	32.1	1.3	0.3	5.1	3.3	6.4	3.6	21.5	5.4	28.5	7.1
C.R.	4	29.1	33.1	2.0	0.3	4.9	3.3	6.9	3.7	22.2	5.6	29.4	7.4
M.C.	4	28.9	32.9	0.9	1.1	6.9	3.8	7.8	4.9	21.1	5.2	28.0	7.0
D.B.	4	42.0	33.8	1.2	0.2	Study II-B		3.5	1.6	4.7	1.8	37.3	9.3
E.R.	4	46.2	40.7	2.1	2.7	10.2	4.6	12.3	7.3	33.9	8.5	33.4	8.4
L.B.	4	44.1	37.2	0.8	0.1	4.0	1.7	4.8	1.8	39.3	9.8	35.4	8.8
B.G.	4	43.6	36.4	1.3	1.2	6.1	2.6	7.4	3.9	36.2	9.1	32.5	8.1
B.K.	4	42.0	33.8	3.2 <sup>1</sup>	1.5	7.9	3.6	11.1	5.1	30.9	7.7	28.7	7.2

<sup>1</sup> Represents 3 days, as 1 day's collection of urine was lost.

differed from these values by 0.8 mg for iron, and by 5.4 mg for copper. The copper of the diets in the present study is much higher than Leverton and Binkley's ('44). This is apparently a regional effect upon the composition of food since this was also found to be true of Nursery School lunches analyzed.

In general, all of the subjects showed an increased retention of both iron and copper when these elements were present in greater amounts in the diet (table 1) although these increases were not consistent. This is in accord with the findings of Leverton and Binkley ('44), that as the intakes of iron and copper increased in the diet, the retention of both elements increased. The total urinary iron ranged from 0.2 to 3.2 mg, and the fecal iron from 2.9 to 10.2 mg. However, the excretion of iron was higher than that of copper in both the urine and feces with a wider range for copper than for iron. These figures indicate that the total excretion of iron was greater than that of copper in urine and in feces.

#### SUMMARY

The daily intakes of iron and copper in food and milk were 6.6 to 13.6 mg, with an average of 9.6, and 6.5 to 13.0 with an average of 8.1 mg, respectively.

There were no consistent increases in the excretion of iron and copper when the intakes increased. In all instances the total excretion of iron exceeded that of copper. However, increased intakes of both iron and copper usually resulted in increased retentions of each.

All of the 17 young college women on self-selected diets were in positive iron and copper balance.

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# SUITABILITY OF THE BOURQUIN-SHERMAN DIET FOR RIBOFLAVIN ASSAYS AND RESULTS OBTAINED IN THE ASSAY OF EVAPORATED MILK

LILLIAN BELLE COSNER AND CECILIA SCHUCK

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TWO FIGURES

(Received for publication December 22, 1947)

The Bourquin-Sherman technic and various modifications thereof are commonly employed in biological assays for riboflavin. In recent years the use of the riboflavin deficient basal diet developed by Bourquin and Sherman ('31) has been questioned. Gyorgy et al. ('34), Street ('41), Lindholm ('38) and El Sadr et al. ('40) have presented evidence of deficiencies in this basal diet of B factors other than riboflavin. As a result of the observations of these workers, other materials have been proposed by them and by Wagner et al. ('40) to supplement or replace the alcoholic extract of wheat used by Bourquin and Sherman to supply the members of the B-complex exclusive of riboflavin.

It is possible that all of the basal diets designed for riboflavin assay fall short of the ideal in that they may not (a) be entirely free from riboflavin, or (b) contain all other nutrients necessary for the rat. While claims of superiority of other basal rations over the Bourquin-Sherman diet are not without support, Day and Darby ('47) have recently published the following statement concerning the latter: "For assays wherein the growth is somewhat limited, and such should be the case in a quantitative riboflavin assay by the rat growth method until the other factors of the diet have been more



carefully studied, growth elicited by a supplement to the Bourquin-Sherman diet may be interpreted as a measure of riboflavin."

The purpose of this investigation was twofold, namely, (a) to investigate further the suitability of the Bourquin-Sherman basal diet for the determination of the riboflavin value of foods, and (b) to obtain a measure of the riboflavin value of evaporated milk based upon the use of this basal diet. Although there have been a number of reports on riboflavin values for fluid whole milk determined by the rat growth assay method, figures for only a few such assays are available for evaporated milk (Todhunter, '32; Henry et al., '40).

#### EXPERIMENTAL PROCEDURE

Forty-eight young albino rats, 21 to 22 days of age, equally divided as to sex, and weighing 40 to 50 gm, were placed on the Bourquin-Sherman basal ration. Thirty-six of these were carried to depletion and 12 were supplemented immediately. The latter were divided into 2 groups, each group consisting of 3 males and 3 females. One group received pure riboflavin in addition to the basal diet, and the other group pure riboflavin plus thiamine, pyridoxine, calcium pantothenate and choline. The riboflavin was administered in a 20% alcoholic solution 3 times weekly in amounts equivalent to 75  $\mu$ g daily. The other vitamins, with the exception of choline, were mixed and likewise administered in a 20% alcoholic solution 3 times weekly. The daily equivalents for these vitamins were as follows: thiamine and pyridoxine, 30  $\mu$ g each, and calcium pantothenate, 150  $\mu$ g. The choline was incorporated in the diet in the amount of 1 gm per 1000 gm of the diet, a procedure recommended by Miller ('43).

The 36 unsupplemented rats were considered depleted when they reached a level where they gained less than 6 gm in 2 weeks. This required from 32 to 34 days. The rats were then divided into 6 groups, each group consisting of 3 males and 3 females. Three of the groups were supplemented with pure riboflavin in a 20% alcoholic solution fed 3 times weekly in

amounts equivalent to 3, 6 and 9  $\mu\text{g}$  daily. A fresh supply of the riboflavin made up according to the method of Wagner et al. ('40) was prepared each week and stored in a dark bottle in the refrigerator. The other 3 groups were supplemented with undiluted evaporated milk in amounts providing presumptive levels approximating those of the pure riboflavin. These estimated quantities were supplied by feeding 3 times weekly a daily equivalent of 0.8, 1.6 and 2.4 ml.

#### RESULTS AND DISCUSSION

The average growth rates of the 2 groups of rats on the Bourquin-Sherman diet supplemented from the beginning are reported as graphs in figure 1. The average gain for a 9-week

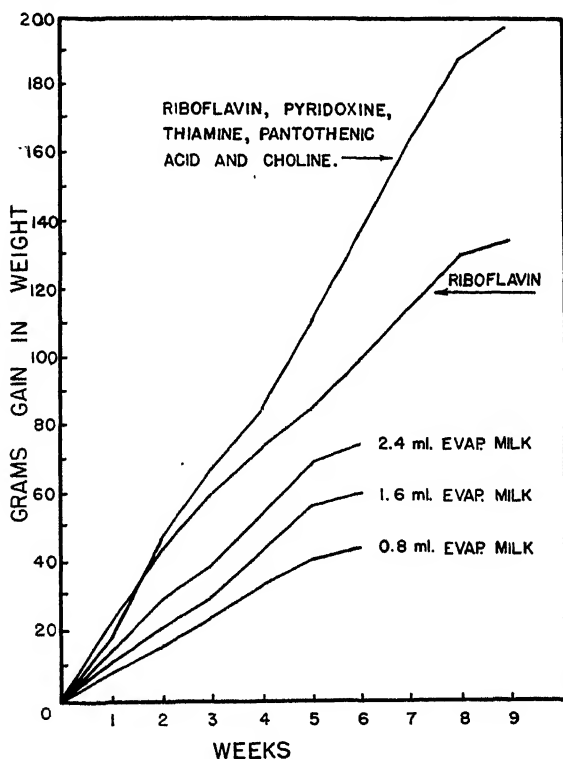


Fig. 1 Gains made on Bourquin-Sherman diet supplemented with evaporated milk; with riboflavin alone; and with riboflavin plus pyridoxine, thiamine, pantothenic acid and choline.

period of the group supplemented with riboflavin alone was 133.7 gm, while the group receiving riboflavin, thiamine, pyridoxine, calcium pantothenate and choline made an average gain of 198.8 gm. The data would seem to indicate that the Bourquin-Sherman diet has B vitamin inadequacies in addition to riboflavin. Further evidence of a deficiency of some

TABLE 1

*Growth response of riboflavin-deficient rats to graded levels of pure riboflavin and evaporated milk as a source of riboflavin.*

DAILY LEVEL OF SUPPLEMENT	NUMBER OF RATS	AVERAGE GAIN PER WEEK (GM)						AVERAGE GAIN	
		1	2	3	4	5	6	For 6-week period	Daily for 6 weeks
<i>µg</i>									
		Pure riboflavin as the supplement							
3	6	8.4	6.5	3.8	6.6	3.8	2.1	31.2	0.74
6	4 <sup>1</sup>	10.8	10.2	8.1	11.0	10.6	3.0	53.7	1.28
9	6	12.3	10.8	11.1	13.9	10.8	5.9	64.8	1.52
<i>ml</i>									
		Evaporated milk as source of riboflavin							
0.8	6	8.1	7.6	7.6	10.3	8.0	3.2	44.8	1.06
1.6	4 <sup>2</sup>	11.7	8.7	7.0	16.0	12.2	3.2	58.8	1.39
2.4	4 <sup>3</sup>	15.3	13.3	10.2	16.9	12.0	5.9	73.6	1.75

<sup>1</sup> A male rat in this group died during the fourth week of the assay period.

<sup>2</sup> A male animal in this group lost weight for a period of 2 weeks, due to an unexplainable illness.

<sup>3</sup> A female in this group, presumably due to animal variation, made an abnormally high weight gain.

The growth rates of the above rats were not included when calculating average gains in weight. To insure an equal number of males and females in each group 1 rat of the opposite sex was eliminated in each case.

nutrient other than riboflavin was the occurrence of slight alopecia in 2 of the rats supplemented with riboflavin alone. Alopecia was also observed in 2 of the rats subjected to riboflavin depletion. One of these rats later received riboflavin supplementation at the 6 µg level, and the other evaporated milk at the 1.6 ml level. The alopecia disappeared in about 2 weeks in the rat receiving evaporated milk, but the one on

the riboflavin supplement remained unchanged throughout the assay period. At the end of the assay period the amount of riboflavin was increased to 200  $\mu\text{g}$  daily with no effect. There was then administered in addition to the riboflavin 90  $\mu\text{g}$  of pyridoxine, with a resulting growth of hair. It has been suggested by Bessey and Wohlbach ('39) and by Clark et al. ('40) that one of the deficiencies of the Bourquin-Sherman diet is pyridoxine, and the observations just recorded give support to this suggestion.

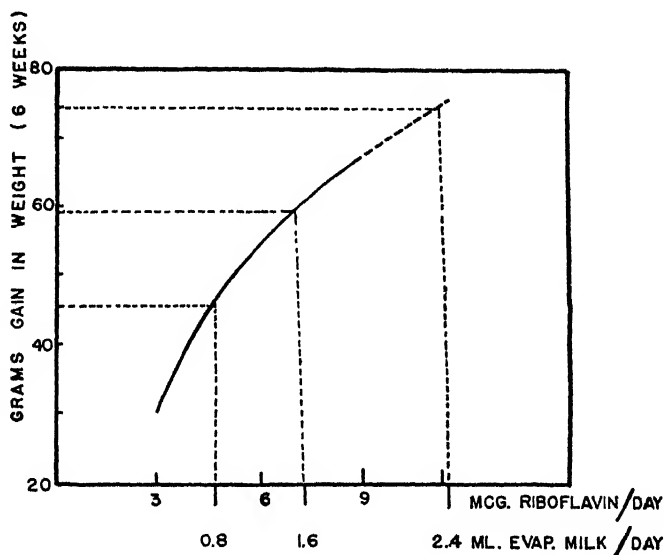


Fig. 2 Curve of response constructed from average gains of rats on graduated doses of pure riboflavin. Dotted lines indicate how curve is used to convert weight gains of rats fed evaporated milk into riboflavin values.

Table 1 shows the growth response of riboflavin depleted rats to graded amounts of pure riboflavin and evaporated milk. The graphic representation of the growth rates on evaporated milk has been included in figure 1. Figure 2 shows the curve of response constructed from the average gains of the riboflavin supplemented rats for a 6-week period. The rate of gain was found to be roughly proportional to the

amounts of supplement fed and there was no overlapping of average weekly gains for the different levels ingested.

In order to interpret the gains on evaporated milk as micrograms of pure riboflavin, they were plotted on the curve of response, and from the points of intersection, perpendicular lines were dropped to the base line and their locations were read directly as micrograms of riboflavin. The riboflavin content of the highest level of milk (2.4 ml) was obtained by extrapolation of the curve, since the growth rate on this level of milk exceeded that of the highest level of pure riboflavin fed. The average of the riboflavin values for the 3 levels of milk was found to be 5.1  $\mu\text{g}$  per milliliter. This is higher than the values for evaporated milk reported by Todhunter ('32) and Henry et al. ('40). However, this study is not wholly comparable with that of the above investigators. Todhunter's work was done before pure riboflavin was available for use as a reference standard, and Henry et al. used a different basal diet. Also the evaporated milk assayed by Henry et al. appears to have been more dilute than the commercial product used in this study. The latter was stated to contain not less than 29.5% total solids.

The riboflavin value obtained for evaporated milk in the present investigation is in close agreement with the upper limits of riboflavin bioassay figures reported for whole fluid milk (Kunerth et al., '37; Lundt et al., '39) when correction is made for the concentration of the evaporated milk. This suggests that riboflavin is not lost in the evaporation processes. In general, the riboflavin values reported for both whole fluid milk and evaporated milk based upon microbiological and fluorometric measurements are lower than those obtained by bioassay (Whitnah et al., '37; Daniel and Norris, '44). This can probably be explained on the basis of deficiencies other than riboflavin in the basal diets used, and the presence in milk of the deficient factors. Evidence has been presented in this study of insufficient amounts of B vitamins other than riboflavin in the Bourquin-Sherman basal diet.

## SUMMARY

An investigation was made which included:

1. A study of the adequacy of the Bourquin-Sherman riboflavin deficient diet with respect to certain other members of the vitamin B complex.

2. The use of the Bourquin-Sherman basal diet in the determination of the riboflavin content of evaporated milk.

The growth rate of a group of rats on the Bourquin-Sherman diet supplemented with riboflavin alone was considerably less than that of a similar group of rats receiving, in addition to riboflavin, supplements of thiamine, pyridoxine, calcium pantothenate and choline. The results indicate a deficiency of the basal diet in one or more of the B vitamins in addition to riboflavin. Some evidence was obtained which suggested that pyridoxine was suboptimal.

In measuring the riboflavin content of evaporated milk, pure riboflavin was used as a reference standard. The value obtained was 5.1  $\mu\text{g}$  per milliliter. This value is higher than that obtained for evaporated milk in earlier rat growth assays employing somewhat different technics. It is, however, in close agreement with the upper limits of riboflavin figures for whole fluid milk assayed by the Sherman-Bourquin method, when the concentration of the evaporated milk is taken into account. This suggests that riboflavin is not lost in the evaporation processes.

In general, recent bioassay riboflavin figures for milk have been found to be higher than those obtained by the microbiological and fluorometric methods. A probable explanation, in the light of the results of this and other investigations, is the presence in milk, in addition to riboflavin, of B factors not present in optimal amounts in the basal diet.

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PROCEEDINGS OF  
THE TWELFTH ANNUAL MEETING OF THE  
AMERICAN INSTITUTE OF NUTRITION

HADDON HALL AND MUNICIPAL CONVENTION HALL,  
ATLANTIC CITY, NEW JERSEY, MARCH 14-19, 1948

COUNCIL MEETINGS

Council meetings were held at Haddon Hall on Sunday and Monday, March 14 and 15. Formal actions of the Council are reported in the minutes of the business meetings.

SCIENTIFIC SESSIONS

The scientific program consisted of 6 half-day sessions of papers grouped according to topic. A total of 65 papers was presented, and 7 were read by title. In addition, the Tuesday afternoon session was devoted to the Joint Session of the Federation.

BUSINESS SESSIONS

Two business meetings were held, one at 10:00 P.M. Tuesday evening, March 16, following the Institute Dinner; the other at 4:30 P.M. Thursday, March 18, following the scientific program. President R. M. Bethke presided at these meetings.

*Tuesday, March 16, 10:00 P.M.* The Secretary gave a brief statement on the Institute membership, noting the death of Fred C. Koch on January 26, 1948. The report of the Treasurer was presented by N. R. Ellis. The Auditing Committee (O. L. Kline and H. R. Bird) reported that the Treasurer's books were in order. The Treasurer's report was approved.

Dr. George R. Cowgill, Editor of *THE JOURNAL OF NUTRITION*, gave a brief report concerning the affairs of the *JOURNAL* as follows:



Beginning with January, 1947, *THE JOURNAL OF NUTRITION* has had an allowance of 120 pages per issue instead of the former 100 pages. The editor had asked The Wistar Institute to make this increase in order to permit more prompt publication of the acceptable material submitted. There seemed to be a definite trend in the direction of greater number of papers being offered. The recommendation to The Wistar Institute was based on the records indicating this trend. This increase meant a change in volume size from one of 600 pages to one containing 720 pages. As usual, 2 volumes were published during the year, namely, volumes 33 and 34; they contained 121 articles. During the year 166 papers were submitted for consideration. The average number of papers per issue was 10, the same as for the previous year; the average number of pages per article was the same as that for the previous year, namely, 12.2 pages.

In keeping with the plan to share, whenever possible, any success of the Journal with the membership of the American Institute of Nutrition, the editor has from time to time discussed with Dr. Edmond J. Farris, Executive Director of The Wistar Institute, whether the subscription rate to members of the society might not be reduced. Whether this could be done was essentially a financial matter. Sometime ago it was decided that if our volume of advertising should be sufficient and should prove to be fairly constant and the number of subscriptions should continue to be sufficient, something along this line might be done. For some time now these conditions appear to have been met. As a result, the Editor proposed recently to The Wistar Institute that, beginning with volume 36, the first issue of which appears in July, 1948, the subscription rate for members of the American Institute of Nutrition be set at one-half the regular rate. This request has been granted. This agreement will remain in effect as long as general income (including the advertising revenue) will be sufficient to offset satisfactorily the cost of printing the Journal.

The Editor's report was approved and the members extended to Dr. Cowgill and the Editorial Board a vote of sincere appreciation for their efforts.

Dr. R. E. Johnson described the organization of various Pathological Registries in the Army Institute of Pathology and proposed that the American Institute of Nutrition sponsor a Registry of Nutritional Pathology, since a request by an interested Scientific Society to the Scientific Director of Registries is a necessary step in setting up such a Registry. Dr. Johnson explained the value of this Registry and pointed out that only a token contribution would be needed from the Institute since the Registries are financed in other ways. After some discussion it was decided that the President should appoint a small *ad hoc* committee (preferably composed of a medical nutritionist, an animal nutritionist, a biochemist, and 1 person familiar with Army organization) to consult with the Scientific Director, American Registry of Pathology, concerning the establishment of a Registry of Nutritional Pathology.

The Secretary reported on correspondence with Dr. Leslie J. Harris who has been empowered by the (British) Nutrition Society to carry on negotiations leading to the establishment of an International Union of Nutrition Societies. Dr. Harris suggested that a Provisional Committee (consisting of 1 or more representatives of each of the 13 countries contacted) should meet in England during the summer of 1948 to explore the situation. The membership expressed approval of sending a delegate to this meeting from the American Institute of Nutrition provided some method could be found for meeting the expenses involved.

The President reported that the Nutrition Foundation had expressed a desire to provide an award of One Thousand Dollars each year to a scientist making an outstanding contribution to nutrition and had asked the Institute to administer the award. The following report was made by the Committee appointed to consider this matter:

*Report of the Special Committee on Rules and Regulations  
Governing the Annual Award Made Available  
by the Nutrition Foundation, Inc.*

The Committee makes the following recommendations:

1. That the American Institute of Nutrition accept with sincere appreciation the offer of The Nutrition Foundation, Inc. to provide annually an award of One Thousand Dollars for outstanding accomplishments in the science of nutrition, and the expenses of the recipient, or recipients, incident to attendance at the annual meeting at which the award is made, and the cost of preparation of an appropriate scroll, the cost of travel and the preparation of the scroll to be within the limit of the sum provided for these purposes.

2. That the award be known as the Research Award of the American Institute of Nutrition.

3. That the award be made in recognition of outstanding accomplishments in the general field of exploratory research in the science of nutrition.

4. That it be made to an investigator who, in the opinion of a Jury of Award, has made the most significant published contribution in approximately the year preceding the annual meeting of the Institute or, who has published a series of contemporary papers of outstanding significance.

5. That, as a general policy, the award be made to 1 person. If, in the judgment of the Jury of Award an injustice would otherwise be done, it may be divided among 2 or more persons.

6. That normally, the preference be given to research workers in the United States and Canada, but that investigators in other countries are not to be excluded from consideration. (Thus, it might be appropriate to give consideration to an investigator who has done outstanding work and who is a resident in, or who is visiting, the United States or Canada for a period of time.)

7. That there be no limitation as to age.

8. That membership in the American Institute of Nutrition is not a prerequisite of eligibility for the award.

9. That the award be made at the annual meeting of the American Institute of Nutrition.

10. That the recipient be presented with a suitably inscribed scroll signed by the President of the American Institute of Nutrition.

11. That if, in the opinion of the Jury of Award, no suitably qualified candidate is nominated in a given year, it shall so report to the President of the Institute and no award shall be made.

12. That an announcement of the award be made each year in the July, October, November and December issues of The Journal of Nutrition, including a request that nominations be sent to the Chairman of the Nominating Committee before January 15 of the year in which the award is to be made.

13. That a Nominating Committee of 3 members be appointed by the President to receive and to make nominations. To maintain a continuity of experience and to provide for rotation of membership, the first committee shall consist of 1 member appointed for a 3-year term, 1 for a 2-year term and 1 for a 1-year term. Thereafter, members shall be appointed for 3-year terms. The member with 1 year yet to serve shall be chairman.

14. That a Jury of Award of 5 members of the Institute be named by the President, in accordance with the provisions stated below, to select a recipient.

To provide for rotation of membership and at the same time to maintain a continuity of experience, the first Jury shall consist of 2 members appointed for 3-year terms, 2 for 2-year terms, and 1 for a 1-year term. Thereafter all appointments shall be for 3-year terms. After a juror has served a term of any length he shall not be eligible for another appointment until after the lapse of 1 year. The President of the Institute shall be Chairman of the Jury but shall not be entitled to vote.

When a vacancy occurs on the Jury, the President shall fill the vacancy by appointment for the unexpired term.

If a juror is nominated for the award, he shall resign his appointment immediately and the President shall fill the vacancy by appointment for the unexpired term.

15. That the Jury of Award use the following procedure in receiving and considering nominations.

Promptly after January 15 of the year in which the award is to be made, the Chairman of the Nominating Committee shall transmit all documents to the Chairman (President of the Institute) of the Jury of Award. The Chairman of the Jury will then transmit the nominations with supporting data to the members of the Jury requesting prompt review and compliance with the following procedure:

The Chairman shall first ask the members of the Jury whether any candidate is worthy of the award. If a majority of the members answer in the negative, no award will be made. If a majority answer in the affirmative, then the Jury shall at once proceed to ballot, each member of the Jury indicating to the Chairman his first, second, and third choice. If a majority of the members of the Jury select as first choice the same person from the list of nominees, such a choice shall be final and that person shall be designated as the recipient of the award. If there is no such agreement, the Chairman shall weigh each first choice as 3, each second choice as 2, and each third choice as 1, and shall total the score of each nominee as obtained by this method. He shall then prepare a list of preferably not more than 7 of those having the highest total score. Balloting shall be continued on the candidates so selected. On each subsequent ballot each member of the Jury shall vote and the Chairman shall eliminate the candidate having the lowest point score until but 2 remain. From these 2 the Committee shall select the recipient. However, if on any ballot any candidate is the choice of a majority of the members of the Jury, he shall be declared elected.

The final decision of the Jury must be made at least 3 weeks before the Annual Meeting of the Institute.

The President of the Institute shall communicate the decision of the Jury to the recipient.

Respectfully submitted,

WALTER RUSSELL, *Chairman*

GEORGE R. COWGILL

VINCENT DU VIGNEAUD

D. W. WOOLLEY

ARTHUR H. SMITH.

The above report was approved by the members.

The Secretary reported that the Executive Committee of the Federation had taken the following actions:

1. Elected Dr. M. O. Lee as Secretary-Treasurer for the coming year. (Dr. Lee has already established a permanent office in the National Academy of Sciences building in Washington.)

2. Decided that the 1949 meeting should be held in Detroit (week of April 18) and the 1950 meeting in Atlantic City.

Also recommended that the meeting be held every other year in Atlantic City.

3. Recommended that the Joint Session of the Federation be held in the evening.

4. Recommended that the Federation assessment be set at Three Dollars per member, all of this to be used in support of *Federation Proceedings*.

5. Recommended that the Federation By-Laws be altered so as to permit the Control Board to elect a Chairman.

6. Recommended that no reprints of the abstracts published in *Federation Proceedings* be made available to the authors.

Items 3, 4, 5, and 6 were approved by the members of the Institute.

The financial obligations of the Institute members for the year July 1, 1948-June 30, 1949 were fixed at Nine Dollars (Five Dollars for *Journal of Nutrition*, Three Dollars for *Federation Proceedings*, One Dollar for Institute of Nutrition dues).

A motion to enlarge the ballot by including space for the suggestion of 5 names for the Nominating Committee was approved.

The members approved the proposition that the Executive Committee should advise its A.A.A.S. council representative to urge that legislation creating a National Science Foundation should specify that the Director should be selected by the President from a list provided by the National Academy of Science.

The Program Committee (consisting of the Secretary and members appointed by the President) was empowered to construct the program of the annual meeting in such a way as to meet any space limitations imposed on the Federation Meeting and to limit the number of papers presented if this move became essential.

It was decided to hold a Dinner annually and that this function should not be restricted to members if adequate facilities were available.

The President appointed Drs. T. H. Jukes and N. B. Guerant as Tellers.

The meeting adjourned at 12:00 P.M.

*Thursday, March 18, 4:30 P.M.* The Tellers reported that the following officers had been elected for 1948-1949:

President, E. M. Nelson  
Vice-President, C. G. King  
Treasurer, N. R. Ellis  
Secretary, J. H. Roe  
Councillor, E. N. Todhunter  
Associate Editors, R. H. Barnes  
P. L. Day  
H. H. Mitchell

The following new members were elected on recommendation of the Council:

Chow, B. F.	Johnston, Frances Ann
Dodds, Mary L.	Leichsenring, Jane M.
Engel, R. W.	McCoy, R. H.
Everson, Gladys	Rusoff, L. L.
French, C. E.	Shaw, J. H.
Frey, C. N.	Todd, W. R.
Heinle, R. W.	Youmans, J. B.
Higgins, G. M.	

The Council was given authority to designate an official representative to the Provisional Committee meeting this summer to consider an International Union of Nutrition Societies, provided financial arrangements for such a delegate could be completed.

The membership voted that the Secretary should send the following resolution to the President of the United States, President of the Senate, and Speaker of the House:

The American Institute of Nutrition hereby records its strong disapproval of the methods employed by the Committee on Un-American Activities of the House of Representatives in publicly implying, without affording proper opportunity for reply, that Dr. E. U. Condon, Director of the National Bureau of Standards, had failed properly to safeguard secrets vital to the safety of the United States. The Committee on Un-American Activities violated basic civil rights in thus making implied accusations not proved by

facts in evidence. The Institute wishes to emphasize that the fabric of cooperation between scientists and their government, so carefully and strongly woven during the war and so important for the welfare and safety of the United States will be seriously weakened if the loyalty and integrity of citizens can be publicly questioned without furnishing acceptable substantiating evidence in conformity with established judicial procedures.

Dr. E. M. Nelson of the Joint Committee on Nomenclature reported that the Committee proposed using the term "folacin" as synonymous with pteroylglutamic acid. The Institute approved the request of the Committee that publicity be given this suggestion before a final decision was reached.

It was announced that the first International Congress on Biochemistry would be held in London in August, 1949.

The Institute gave a hearty vote of thanks to Drs. Lee and Chambers for their fine efforts in organizing and arranging the meeting.

President Bethke appointed the following Nominating Committee for 1948-1949:

H. G. Day, Chairman  
H. Hunscher  
H. J. Deuel  
E. W. Crampton  
F. J. Stare

The meeting adjourned at 5:30 P.M.

#### DINNER AND PRESENTATION OF AWARDS

The Institute held its annual dinner on Tuesday evening, March 16, at the Jefferson Hotel. Dr. Frank G. Boudreau gave a very interesting and stimulating talk on the opportunities for improvement of world conditions through better nutrition.

The Mead Johnson and Company Prize was presented to Dr. Fritz Lipmann and the Borden Award to Dr. Charles A. Cary.

Respectfully submitted,

H. E. CARTER, *Secretary*  
American Institute of Nutrition





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